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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our most significant findings during the second year of work included the following:
1. The PRL-humanized mice synthesize and secrete hPRL that is fully biologically active at the human PRL receptor as evidenced by activation of STAT5 in human breast cancer cells.
2. Our "proof of concept" experiment in which human breast cancer xenografts were studied in hosts bearing anterior pituitary grafts under the renal capsules, supports the hypothesis that elevated circulating hPRL leads to increased growth of human breast cancer tumors. The rate of growth of xenografts was greatest in hosts bearing hPRL-secreting pituitary grafts as compared to those bearing mPRL-secreting or PRL-knock out pituitary grafts.
3. We now have the PRL-humanized mice on the immunodeficient SCID background and are planning multiple collaborations with other investigators.

15. SUBJECT TERMS
PRL (prolactin), xenografts

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Summary of work completed in Year 2

INTRODUCTION

Prolactin (PRL) has been shown to stimulate the proliferation and metastatic mechanisms of breast cancer cells. Moreover, epidemiological findings demonstrate that PRL is a risk factor for the development of breast cancer. Yet, the role of PRL in human breast cancer remains poorly understood because there is currently no animal model appropriate for its study. This is due to the fact that rodent PRL does not activate the human PRL receptor (hPRL-R). In our studies, we proposed to create a mouse model that is compatible for the study of prolactin in human breast cancers by developing mice that make and secrete prolactin that is structurally identical to human prolactin. These mice will enable us to test the hypothesis that elevated PRL levels stimulate progression of tumor growth and metastasis in human breast cancer cell xenografts (Task 1), generate and characterize a PRL mouse model for breast cancer xenograft experiments (Task 2), and test the hypothesis that PRL-receptor positive breast cancer xenografts will be sensitive to physiological changes in endocrine and paracrine PRL (Task 3).

REPORT

Task 1 was to test the hypothesis that elevated hPRL will stimulate progression of tumor growth and metastasis in human cancer cell xenografts.

Task 1a involved xenografting MCF-7 human beast carcinoma cells into the fat pads of immunodeficient mice. These mice ("hosts") were divided into three groups based on circulating PRL levels: all three groups received ectopic (under kidney capsule) grafts of anterior pituitary (AP) glands, a technique commonly used to produce sustained, elevated levels of circulating PRL. These three groups included the following:

1) Hosts with ectopic grafts of AP from wild type mice (PRL^{wt}) – to produce elevated levels of serum mPRL;
2) Hosts with ectopic grafts of AP from mPRL knock-out mice ($PRL^{-/-}$) – these mice have normal levels of mPRL produced by the host animal;
3) Hosts with ectopic grafts of AP from hPRL transgenic mice on the mPRL-knockout background – to produce elevated levels of serum hPRL

As discussed in the previous annual report, the MCF-7 cell grafts did not grow well as evidenced by lack of palpable tumors in the xenografting sites. Since that report, we have performed histological analyses of several of those sites and confirmed that the transplanted cells did not grow and, in many cases, did not survive. This was a possibility that we considered in our proposal - that the MCF-7 cells are non-invasive and grow slowly. We encountered another problem when we analyzed the hosts' blood samples for PRL levels. The data indicated that several of the ectopic AP grafts were not functional. Subsequent histological analysis indicated that some of the grafts were gone and there were substantial adhesions at the site of grafting. This was most likely due to too much trauma to the renal capsule at the graft site – the fragile capsule splits and fails to contain the AP tissue. Although the technician had been trained extensively, practice is what makes these surgeries go well, so I decided to perform them myself for the next set of experiments (see Task 1b, below).

In the meantime, however, these setbacks provided us time to reassess our strategy and also considered the possibility that although our hPRL transgenic mice do produce hPRL (as detected *immunologically*) and that it is secreted both *in vivo* and *in vitro*, it may undergo some type of post-translational processing that renders it less effective at the human PRL receptor. As reported last year, we demonstrated that it is biologically active on the mouse PRL receptor, but this was no guarantee that it would activate the hPRL-R. We felt that it was imperative, before embarking on further expensive transplant studies, to verify that the hPRL produced by the transgenic APs was biologically active at the hPRL-R.

Briefly, extracts of APs from $hPRL^{+}$ mice were used to treat the human mammary carcinoma cells, T47D cells. Extracts from $mPRL^{wt}$ and $mPRL^{-/-}$ APs were also used as negative controls, while recombinant hPRL was used as a positive control. The phosphorylation of STAT5 was measured as an index of hPRL-R activation. STAT5 transcription factors have been identified as critical mediators of the biological activity of PRL and its receptors (Sultan *et al.* 2005). This study demonstrated that the hPRL produced by the transgenic mice is indeed biologically active at the hPRL-R. These data are presented in Figure 1 and the means of replicate analyses are summarized in Figure 2. This experiment will be added to the characterization of our humanized PRL mouse model (manuscript in preparation).

Task 1b: Xenografts of T47D cells in AP-grafted immunodeficient hosts.

After determining that the hPRL produced in transgenic mice is fully active at the hPRL-R, we embarked on Tasks 1b, using similar experimental approaches as Task 1a (immunodeficient mice as hosts; use of ectopic pituitary grafts for altering circulating PRL levels; orthotopic and subcutaneous xenografts). However, these studies used more aggressive, tumorigenic cell lines of human breast carcinoma cells: T47D cells. Hosts were assessed each week for 8 weeks for palpable tumors and such growths were measure with a micrometer. At the end of 8 weeks, the mice were euthanized and samples collected. These included: serum for determination of hPRL and mPRL; T47D xenografts and surrounding tissue; AP grafts and surrounding tissue; samples of lung, bone, lymph nodes, and mammary gland tissues. The AP grafting procedures were very successful as evidenced by the circulating levels of hPRL and mPRL (Figure 3). The three groups had the predicted outcomes of the AP grafts. Groups 1 and 2 had supraphysiological levels of mPRL and hPRL, respectively, due to unregulated secretion of PRL from the ectopic AP grafts. Group 3 had normal levels of mPRL. Of course, Groups 1 and 3 had non-detectable levels of hPRL, since the hPRL gene was not in either of those donor tissues. Group 2 had significantly lower levels of mPRL, demonstrating effective negative feedback of the hPRL produced by the AP grafts on the secretion of the endogenous mPRL of the hosts. Again, this is evidence of the biological activity of the hPRL on the *mouse*PRL-R.

The initial data obtained on the growth of orthotopic human breast cancer xenografts in these hosts is very encouraging. Gross measurements of tumor volume were calculated from two measures of diameter made on different axes, performed once each week. demonstrate a significant increased rate of growth in Group 2, whose hosts had elevated circulating hPRL (Figure 4). At the end of the study, when the tissues were collected, we removed the xenografts and fixed a small piece for histological analysis and froze the remainder for RNA or protein analysis. The remaining tissues were fixed for histology or ICC to determine presence of metastases. Although the funding from the DOD has finished, we are using internal funds to complete these analyses. We have chosen to perform Western blot on the xenografts for the hPRL-R and the apoptosis regulating factor, Bcl-2, both of which have been shown to be increased by overexpression of hPRL in human breast cancer cells (N. Ben-

Jonathan, personal communication). Evidence supports the idea that hPRL may function as a survival factor (anti-apoptotic) in human breast cancer cells, contributing both to growth of tumors and to chemotherapy resistance of breast cancer (LaPensee and Ben-Jonathan, 2010).

Task 2: Task 2 was to complete the generation and characterization of the humanized PRL mouse model as hosts for xenograft studies and to complete the crossing onto an immunodeficient background.

Characterization of PRL-humanized mice: As described in the previous annual report, we completed the cross-breeding of our humanized PRL mice (hPRL^{+}) onto the mouse PRL knock-out (mPRL$^{-/-}$) background. In that report, we presented the data demonstrating tissue expression of hPRL *in the human pattern* and secretory regulation of pituitary hPRL and extrapituitary hPRL by known physiological regulators. The expression of hPRL also rescues the reproductive deficits in the female mPRL$^{-/-}$ mice.

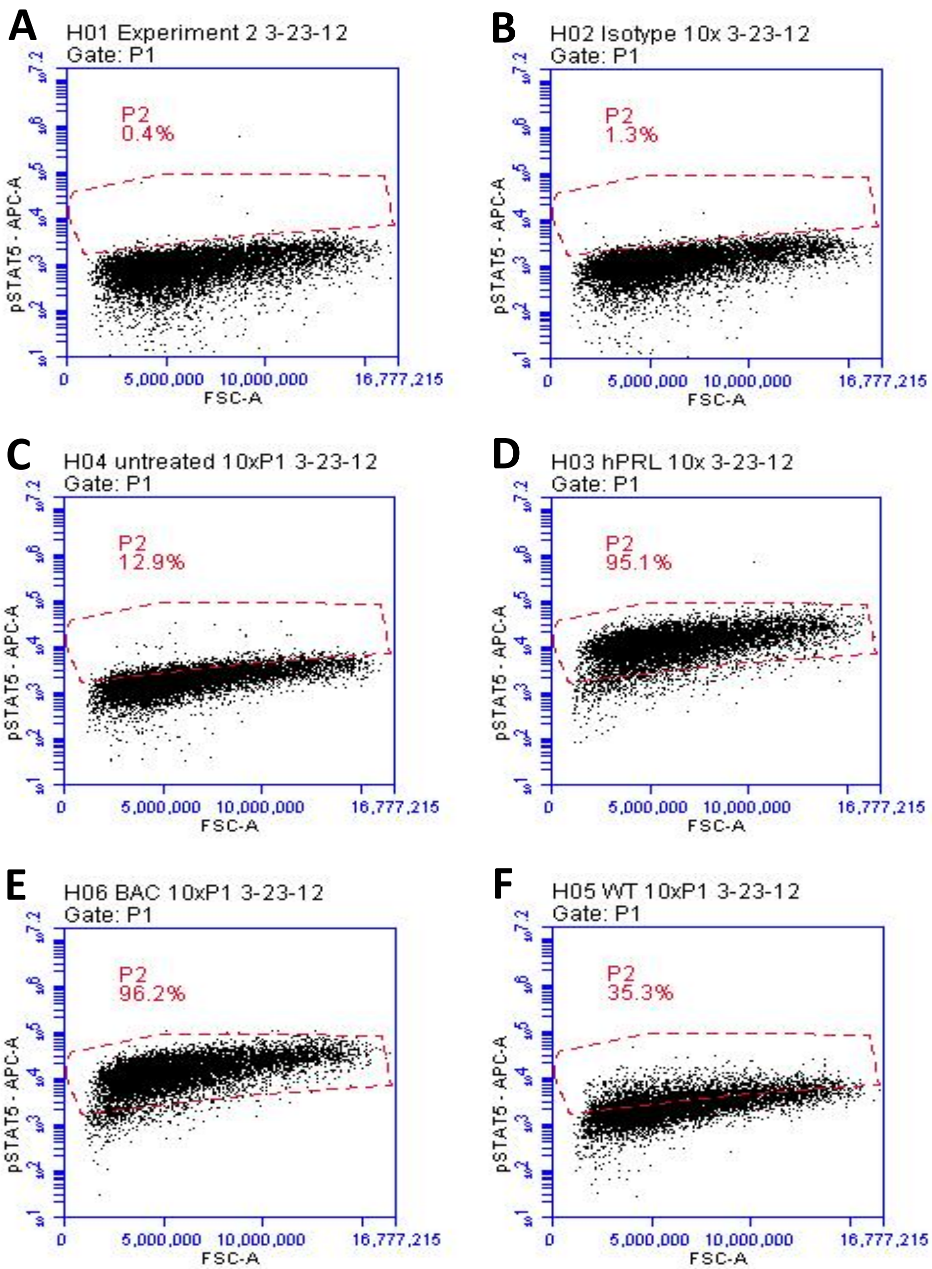
Not mentioned previously is that two lines of hPRL^{+} mice were eventually generated, each from a different founder animal (BAC-h8 and BAC-h30). This was very important (see below) since incorporation of the transgene into the mouse genome was not directed. More than one line is needed to verify that any phenotype is due to expression of the transgene and not disruption of endogenous genes at the insertion site(s). All of the characterizations described above were performed in both lines with the same findings. This included mice carrying both the transgene for hPRL and the endogeneous gene for mPRL, as well as mice carrying the transgene for hPRL on the mPRL$^{-/-}$ background. Some of these data were presented at the FASEB Science Research Conference on "The Growth Hormone/Prolactin Family in Biology and Disease" in July, 2012. Several participants inquired as to whether we had determined the number of copies of transgene incorporated in our mouse lines. We had not, but agreed that this was important information. Therefore, we did perform a FISH (fluorescent *in situ* hybridization) analysis of both lines to determine the copy number of the transgene. We found that the BAC-h8 line has a single copy of the transgene and the BAC-h30 line has two copies (Figure 5).

Breeding of the hPRL^{+} mice onto an immunodeficient background: As discussed in last year's report, we changed our original plans to cross our mice onto the NOD-SCID background, due to the very slow progress of the breeding which required incorporation of the NOD and SCID mutations in both alleles while also introducing the hPRL gene and eliminating the mPRL gene from the NOD-SCIDs. With the large body of work using SCID mice as xenograft hosts for human breast cancer cells, we chose to cross the mice onto the SCID background alone. As predicted, generation of these lines proceeded more quickly and we produced a colony of mice from the BAC-h8 line that contains the SCID mutation on both alleles, null mutation of the mPRL gene (both alleles) and expression of the hPRL transgene (hPRL$^{+/?}$). However, once we had accomplished this we ran into a problem. Mice that had the transgene and the SCID mutation on *both* alleles were runts and had a physical problem with their hind legs (so weak that the mice tended to drag them). This disorder did not rectify as the pups grew and it became clear that these animals were not going to be useful. So, we began again with the BAC-h30 line. We now have this transgenic line on the homozygous SCID background with none of the problems (small birth size, physical problems) observed in the BAC-h8 line. We assume that this is related to the insertion sites of the transgene. While it is not a problem in animals without the SCID mutation or with the SCID mutation in only a single allele, somehow the insertion site of the BAC transgene in the BAC-h8 line is causing a developmental problem when both alleles contain the SCID mutation. It is not due to the expression and secretion of the hPRL since the BAC-h30 line has none of these problems.

As mentioned in our previous annual report, as colleagues have heard about the characterization of our model, we have received numerous requests for collaborations. Our model is seen as a true breakthrough for preclinical studies on PRL-sensitive human cancers, especially breast cancer. I mentioned our collaborations with Dr. Nira Ben-Jonathan (University of Cincinnati) and Dr. Maria Diakonova (University of Toledo) and presented some of their data in the last report. Dr. Ben-Jonathan, with me as a Co-Investigator, submitted a proposal to the NIH that utilized our mice. It was not funded, but was reviewed and we are working on a revised application. Since then, we have been contacted by Dr. Carrie Shemanko, Associate Professor at the Southern Alberta Cancer Research Institute, University of Calgary, and Dr. Shrikant Anant, Professor and Associate Director of Cancer Prevention and Control at the University of Kansas Cancer Center. We will be sharing our PRL-humanized mice with both of these investigators.

SUPPORTING DATA

Supporting data obtained in Year 2 are presented on the following pages (7-11).



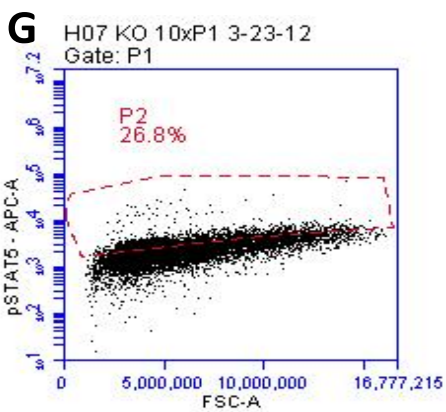


Figure 1. Flow Cytometric Analysis of Stat5 Activation in Human Breast Cancer T47D Cells. Following 16 hrs of serum deprivation, T47D cells were treated for for 20 min (37ºC) with one of the following: **A-C**, control (vehicle); **D**, recombinant hPRL (10 nM); **E**, extract from BAC APs (10 nM hPRL, as determined by RIA); **F**, extract from WT APs (10 nM mPRL, as determined by RIA); **G**, extract from PRL-/- APs (equivalent protein concentration). At the end of the 20 minutes the treatment medium was removed, cells were resuspended, and ice-cold 4% paraformaldehyde. Fixed cells were permeabilized and labeled with Alexa Fluor 647-conjugated anti-Stat5(pY694) (**C-G**), or control anti-IgG2a (κ isotype, **B**), or left unlabeled. Fluorescent labeling was analyzed by flow cytometry.

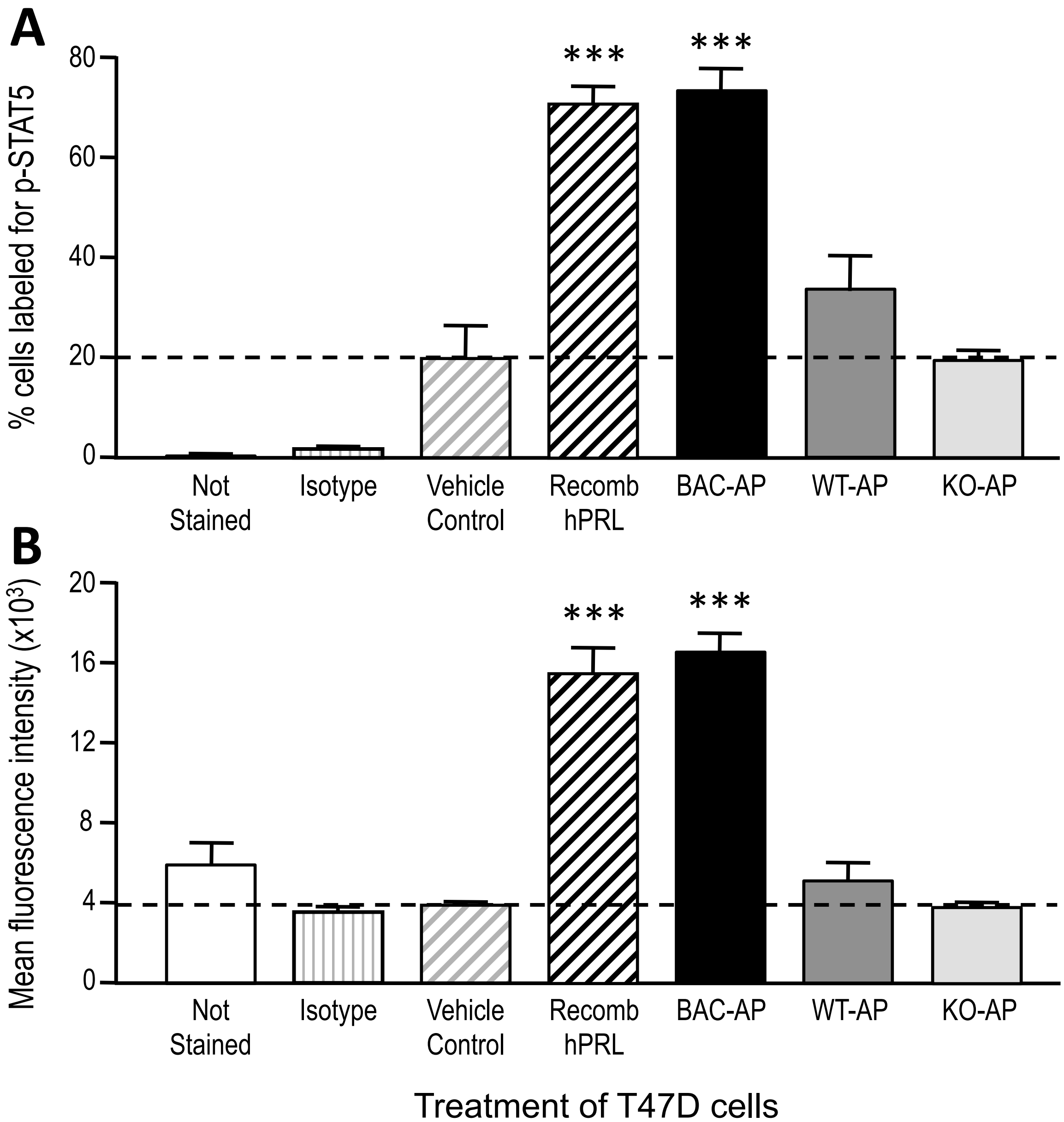


Figure 2. Summary of Flow Cytometric Analysis of Stat5 Activation in Human Breast Cancer T47D Cells. See legend to Figure 1 for description of experiment. Flow cytometric analysis of fluorescent labeling measured the percent of T47D cells labeled (**A**) and the mean fluorescence intensity (degree of labeling) of those positive cells (**B**). The cells not stained or stained for κ isotype served as technical controls (two bars on the left of each graph), while the remaining groups were all stained for phosphorylated Stat5. Vehicle-treated cells served as the biological control (values indicated by dashed lines). Extracts of WT or mPRL$^{-/-}$ (KO) APs did not increase either the percent of cells labeled or the mean fluorescence above vehicle-treatment. Recombinant hPRL (10 nM) increase both parameters approximately 4-fold over vehicle treatment and extracts of APs from hPRL^{+} mice (10 nM hPRL, determined by RIA) were equally effective.

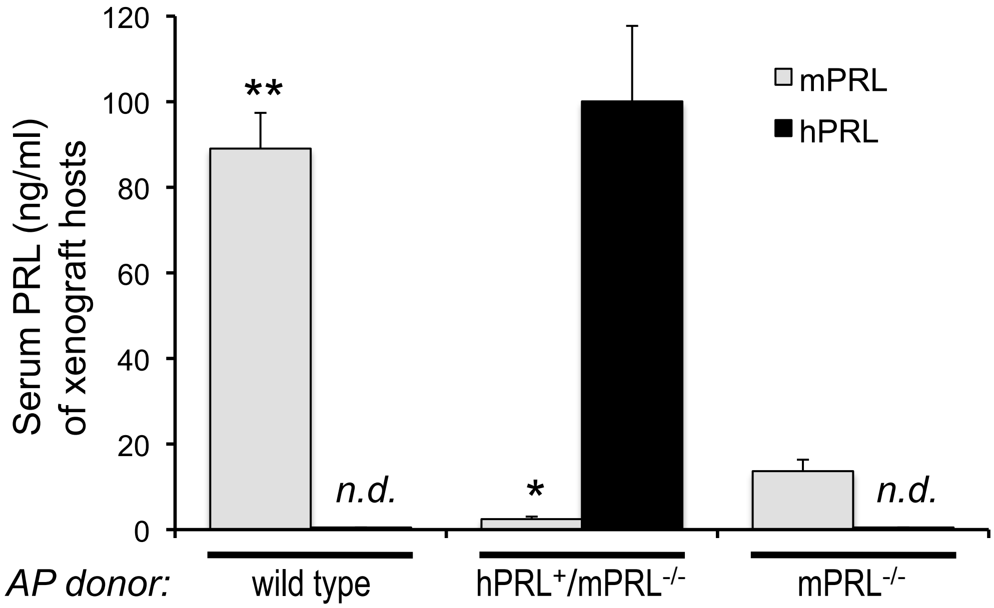


Figure 3. Serum PRL levels in xenograft hosts bearing anterior pituitary (AP) grafts. Levels of mPRL and hPRL were measured in serum samples using species-specific homologous RIA. Mice received two AP grafts (one gland under the capsule of each kidney) from one of three donor types: 1) wild type mice ($mPRL^{+/+}$; no hPRL); 2) PRL-humanized mice ($hPRL^{+/?}$; no mPRL); or 3) PRL-knockout mice ($mPRL^{-/-}$; no hPR). All mice received orthotopic xenografts of human T47D breast cancer cells in the right MG#4. Human PRL was not detectable (n.d.) in hosts bearing wild type or $mPRL^{-/-}$ AP grafts. Serum mPRL was greatly elevated in hosts bearing wild type AP grafts as compared to those with $mPRL^{-/-}$ AP grafts, while serum mPRL was significantly lower in hosts bearing $hPRL^{+}$ AP grafts. The former is due to unregulated secretion of mPRL from the wild type ectopic grafts. The latter is due to suppression of endogenous mPRL by feedback of elevated hPRL produced by the ectopic grafts.

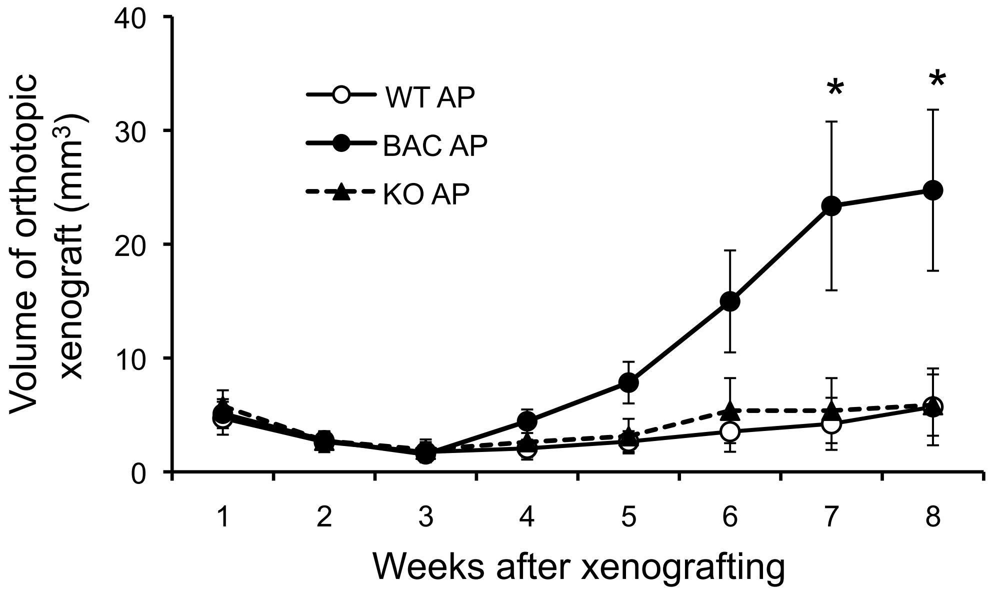


Figure 4. Growth of T47D xenograft tumors in hosts bearing anterior pituitary (AP) grafts. Mice received two AP grafts (one gland under the capsule of each kidney) from one of three donor types: 1) wild type mice (WT, $mPRL^{+/+}$; no hPRL); 2) PRL-humanized mice (BAC, $hPRL^{+/?}$; no mPRL); or 3) PRL-knockout mice (KO, $mPRL^{-/-}$; no hPR). All mice received orthotopic xenografts of human T47D breast cancer cells in the right MG#4. Tumor volume was calculated from two diameter measurements made with digital calipers every week following xenografting.

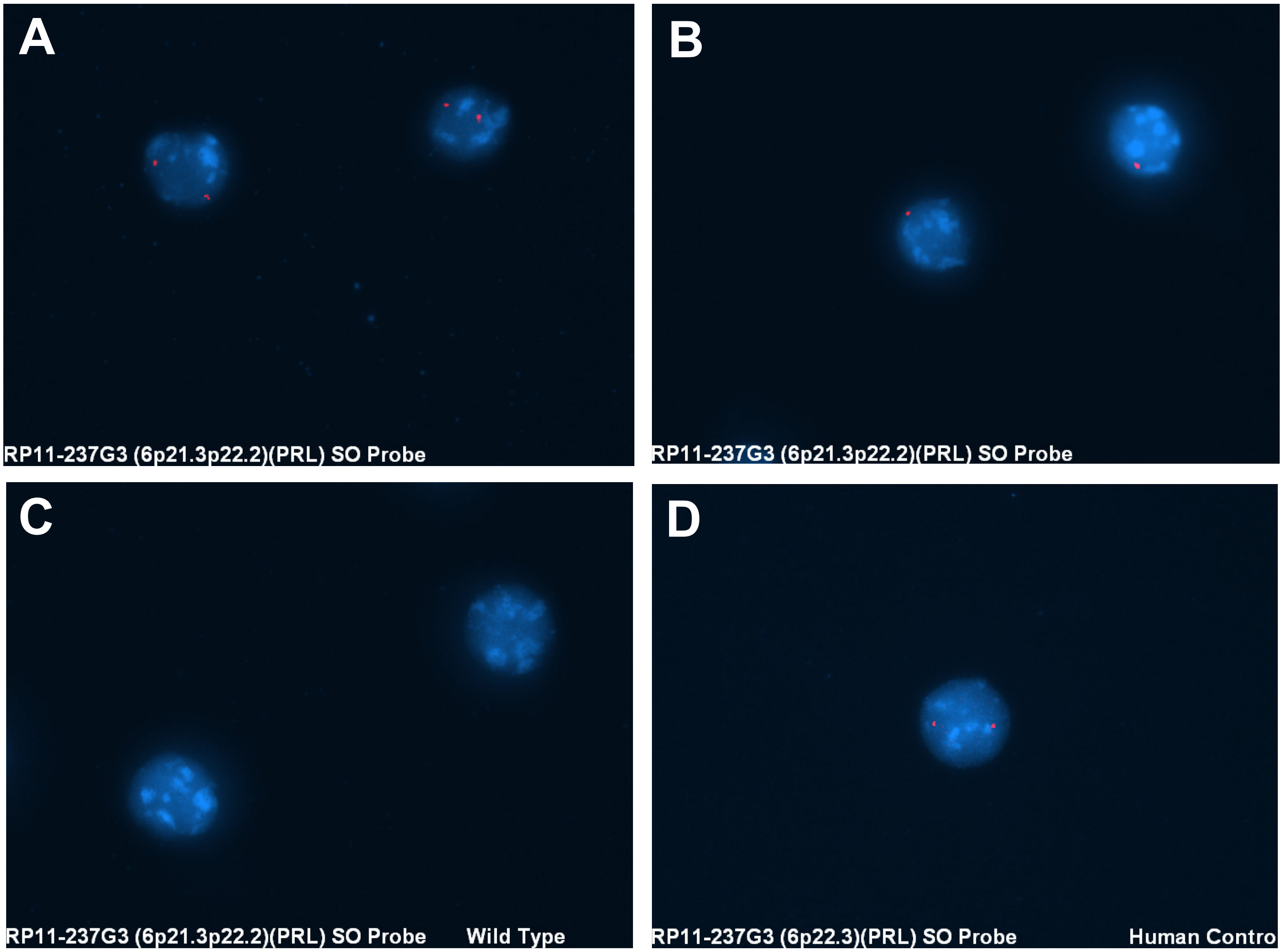


Figure 5. Flourescence *In Situ* Hybridization (FISH) analysis of the PRL-humanized mouse lines. Mouse blood samples were processed according to routine cytogenetic procedure and interphase FISH analysis of mouse samples were performed using a probed targeted to the human PRL gene. One hundred cells per sample were analyzed using fluorescent microscopy. **A**. Cells from the BAC-h30 transgenic line showed two copies of the hPRL gene. **B**. Cells from the BAC-h8 transgenic line showed one copy of the hPRL gene. **C**. Cells from wild type mice were negative for the hPRL gene. **D**. The probes appropriately hybridized to two alleles in human cells.

Table 1

Lab ID #	Sample ID	Sample Info	No. of Cells Analyzed	Signal Pattern
ZO-12-0009	H406	wild-type	100	No probe signals
ZO-12-0010	H344	BAC-h8 line	100	One probe signal
ZO-12-0011	H457	BAC-h30 line	100	Two probes signals

KEY RESEARCH ACCOMPLISHMENTS in Year 2

- We have successfully generated a **novel set of mouse strains that have been humanized with regard to the structural gene, tissue expression and secretory patterns of human PRL**. These models will fill a void in the repertoire of research tools currently available, not only for the direct study of PRL, but for the *comprehensive* study of human breast cancer since they will produce, in physiological patterns, the three principal hormones that direct mammary gland growth and differentiation: estrogen, progesterone, *and PRL.*
- The expression and production of hPRL completely rescued the reproductive deficits characteristic of mPRL-knockout mice. This is of great interest to basic scientists in reproduction and offers, for the first time, a superior model in which the **physiology of extrapituitary PRL** can be studied.
- Moreover, these mice exhibit **induction of hPRL production in peripheral immune tissues** in response to an immune challenge. This is *not* observed with mPRL. This is significant because inflammatory mechanisms play an important role in the metastasis of cancers. Although there is substantial evidence that PRL is involved in immune responses in human tissues, this has not been observed in murine models. This difference may be one reason why treatments developed in mouse models do not translate well to humans, again making our mice superior models for breast cancer research.
- Our "proof of concept" experiment, using ectopic anterior pituitary grafts to manipulate circulating mPRL or hPRL in immunodeficient mice with human breast cancer xenografts, demonstrates that **human breast cancer cells *in vivo* do respond to hPRL with accelerated rate of growth**.

REPORTABLE OUTCOMES

Abstracts/Scientific Presentations (in Appendix):

- Gregerson KA, Christensen HR, Buckley AR, Horseman ND 2011 "Human-Compatible Animals Models for Preclinical Research on Prolactin in Breast Cancer", 2011 BCRP Era of Hope Meeting, August 2-5, Orlando, FL.
- Christensen HR, Horseman ND, Gregerson KA 2012 "Biological Characterization of Mice Expressing the Human Prolactin Gene", 2012 FASEB SRC on The GH/PRL Family in Biology and Disease, July 8-13, Snowmass Village, CO.

Manuscript in preparation (in Appendix):

- Christensen HR, Murawsky MK, Horseman ND, Willson TA, Gregerson KA "Completely Humanizing Prolactin Rescues Infertility in Prolactin Knockout Mice and Leads to Human Prolactin Expression in Numerous Extrapituitary Mouse Tissues", to be submitted to *Endocrinology*.

Degrees earned that were supported by this award:

- Heather Christensen, Ph.D. Systems Biology and Physiology, University of Cincinnati, awarded July, 2011. *Thesis title:* "Molecular and Integrated Systems Physiology of Prolactin".

Current position: Assistant Professor, tenure-track, College of Mount St. Joseph, Cincinnati, OH

Funding applied for based on work supported by this award:

- NIH R-01 "Blockade of Prolactin Signaling in Breast Cancer", Nira Ben-Jonathan, P.I.; Karen A. Gregerson, Co-Investigator. *scored, but not funded; in revision*

Collaborations initiated based on work supported by this award:

- Dr. Nira Ben-Jonathan, University of Cincinnati
- Dr. Maria Diakanova, University of Toledo
- Dr. Carrie Shemanko, Southern Alberta Cancer Research Institute, University of Calgary
- Dr. Shrikant Anant, University of Kansas Cancer Center

CONCLUSION

We have successfully accomplished the goals set out in our original proposal: first, to generate a novel set of mouse strains that have been humanized with regard to the structural gene, tissue expression and secretory patterns of human PRL; and second, to complete a "proof-of-concept" experiment in which we could demonstrate that elevated circulating levels of hPRL in an animal model would promote growth of human breast cancer tumors. These models will fill a void in the repertoire of research tools currently available, not only for the direct study of PRL, but for the *comprehensive* study of human breast cancer since they will produce, in physiological patterns, the three principal hormones that direct mammary gland growth and differentiation: estrogen, progesterone, *and PRL.*

We were not able to begin using our model as hosts for xenografts studies because of the time it took for breeding onto the SCID background and eliminating the mPRL gene again. In addition, we had to begin the breeding over when the BAC-h8 line exhibited developmental problems when the SCID mutation was present in both alleles. However, we have these mice in hand now and have been receiving requests to provide them to other researchers in human breast cancer.

We have high expectations for the usefulness of these novel strains of mice for investigations in both human breast cancer and the physiology of extrapituitary PRL. The fact that the hPRL gene (and not the mPRL gene) is induced in peripheral immune tissues following an immune challenge, may be an additional advantage of these mice. Inflammatory mechanisms play an important role in the metastasis of cancers. The differences in PRL expression in the immune systems of mice *versus* humans may be one reason why treatments developed in mouse models do not translate well to humans.

REFERENCES

Sultan AS, Xie J, LeBaron MJ, Ealley EL, Nevalainen MT & Rui H 2005 Stat5 promotes homotypic adhesion and inhibits invasive characteristics of human breast cancer cells. *Oncogene* **24** 746–760.

LaPensee EW, Ben-Jonathan N 2010 Novel roles of prolactin and estrogens in breast cancer: resistance to chemotherapy. *Endocr Relat Cancer* **17** R91-107.

Human-compatible Animals Models for Pre-clinical Research on Prolactin in Breast Cancer

H. R. Christensen[2], A.R. Buckley[1], N.D. Horseman[2], K. A. Gregerson[1,2]

[1]James L. Winkel College of Pharmacy and [2]Dept. of Molecular and Cellular Physiology, University of Cincinnati, Cincinnati, OH 45267

INTRODUCTION

Greater than 90% of all human breast carcinomas express prolactin (PRL) receptors. Moreover, recent prospective and epidemiological studies have demonstrated elevated serum prolactin as a major risk factor for breast cancer, independent of estrogen and progesterone[1]. Yet, a suitable small animal model for studying human breast cancer does not currently exist because these models do not produce an effective ligand for human prolactin receptors (hPRL-R)[2].

RATIONALE

Using a bacterial artificial chromosome (BAC) containing the entire human PRL (hPRL) gene, we have created a set of mouse strains that synthesize and secrete PRL that is identical both in structure and in tissue expression pattern to hPRL[3]. The identical structure is required to bind to and activate the hPRL-Rs in xenografted human cells. The pattern of expression is critical for determining the influence of both endocrine (serum) hPRL and paracrine (local) hPRL in the growth and metastatic behavior of human breast cancer cells *in vivo*.

HYPOTHESIS

We hypothesized that hPRL-BAC mice would have the human pattern of hPRL expression and that the hPRL would respond to physiologic stimuli.

Moreover, we hypothesized that hPRL would be completely functional in mice and would rescue the reproductive deficits seen in mPRL knock-out ($mPRL^{-/-}$) females.

REFERENCES

[1]Tworoger, S.S., *et al.* 2004. *Cancer Res 64:* 6814-6819.

[2]Utama, F.E., *et al.* 2006. *J Endocrinol 188:* 589-601.

[3]Semprini, S., *et al.* 2009. *Molec Endocrinol 23:* 529-538.

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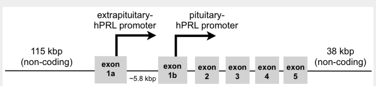
SPECIFIC AIMS

- Generate transgenic mice that express hPRL in a human pattern (in both pituitary and extrapituitary tissues).
- Demonstrate that the hPRL peptide is synthesized and secreted in the mice and regulated in a physiological fashion.
- Cross the hPRL-BAC mice onto the $mPRL^{-/-}$ background and determine if the hPRL rescues the reproductive phenotypes (infertility; alactation) of $mPRL^{-/-}$ females.

METHODS

Transgenic mice: The RP11-273G3 BAC was purchased from Children's Hospital Oakland Research Institute. This BAC is part of human chromosome 6 and contains the entire hPRL gene plus 115 kbp upstream and 38 kbp downstream of the gene locus (*see below*). Purified BAC DNA was provided to the Animal Models Core at Univ. of North Carolina for pronuclear microinjection into mouse oocytes.



Genotyping: Mice carrying the transgene were identified by PCR of genomic DNA identifying unique segments of the BAC at the 5' and 3' ends as well as within the hPRL coding region.

mRNA expression: Whole tissue samples were dissected from genotype-positive BAC mice, flash frozen in liquid nitrogen and stored at -80C until analyzed. Frozen tissues were homogenized in TRIzol and RNA extracted using standard protocol. Total RNA was reverse-transcribed and the cDNA used as the template for PCR using primers specific to mPRL or hPRL.

PRL measurement: Serum, culture medium, and tissue homogenates were assayed for PRL content using double-antibody RIA with reagents from Dr. A.F. Parlow. Primary antisera were specific for either hPRL or mPRL.

Anterior pituitary (AP) cell cultures: AP glands were rapidly dissected and cells dissociated in 0.2% trypsin for 15 min at 37°C. Following washes in Hank's buffer, cells were plated in 12-well dishes and cultured in DMEM + 10% horse serum overnight or for 6 days.

Animal studies: To test hPRL responsiveness, BAC-hPRL mice were subjected to various treatments and physiological challenges. (*See figure legends for details.*) BAC-hPRL mice were cross bred with mPRL-/- mice to produce mice expressing *only* hPRL.

RESULTS

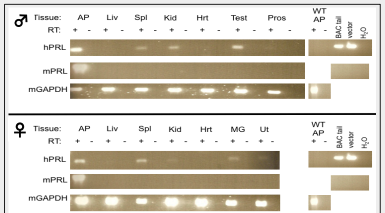


FIGURE 1: hPRL mRNA is expressed in human-like pattern in ♂ and ♀ hPRL-BAC mice.

RNA isolated from various tissues was analyzed by RT-PCR. mPRL transcript was detected in AP only, while hPRL was detected in AP, spleen (Spl), Kidney (Kid), mammary gland (MG), uterus (Ut), testis (Test) and prostate (Pros). *Note specificity of primers in WT AP & BAC vector.*

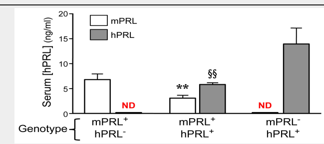
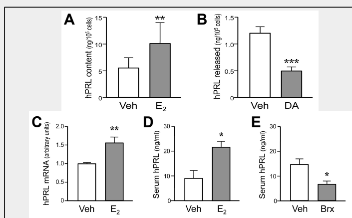


FIGURE 2: hPRL is secreted into the circulation and contributes to feedback regulation in hPRL-BAC mice.

Serum was collected from unstressed female mice expressing endogenous mPRL, or transgenic hPRL, or both. Serum was analyzed in mPRL- and hPRL-specific RIAs. **$p<0.01$ *vs* $mPRL^{+}/hPRL^{-}$; §§$p<0.01$ *vs* $mPRL^{-}/hPRL^{+}$.



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In vitro studies. Primary cultures of pituitary cells from ♀ hPRL-BAC mice were treated: **A,** 6 days with E_2 (80pg/ml) or vehicle, then cells collected; or **B,** 60 minutes with DA (100 nM), then medium collected. In vivo studies. Female hPRL-BAC mice were given: **C** and **D,** s.c. pellets of E_2 or vehicle, and killed14 days later (AP and serum collected); or **E,** s.c. injection of the DA agonist, bromocriptine (Brx), killed 90 min later and serum collected. Sera, cells and media were assayed for hPRL in RIA. AP hPRL mRNA was measured by qRT-PCR. *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs* vehicle control.

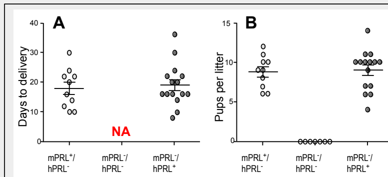

FIGURE 4: hPRL expression in female mice rescues the infertility phenotype that occurs with deletion of the endogenous mPRL gene.

A, Female mice were housed with proven sires and the day of vaginal plug was counted as day 0. *Note: No $mPRL^{-/-}$ mice without hPRL had litters.*
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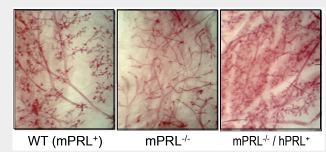

FIGURE 5: hPRL expression in female mice rescues the mammary gland phenotype that occurs with deletion of the endogenous mPRL gene.

Number 4 mammary glands were dissected from nulliparous, 3-month-old female mice and prepared as whole mounts. *Note: Ducts in MGs from $mPRL^{-/-}$ mice (middle panel) have fewer side-branches than WT (left panel) and no alveolar development. Side branching and alveolar development appear normal in hPRL-BAC mice on the $mPRL^{-/-}$ background (right panel).*

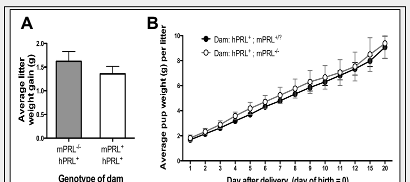

FIGURE 6: hPRL expression in female mice rescues the alactation phenotype that occurs with deletion of the endogenous mPRL gene.

A, Average pup weight gain in a "weigh-suckle-weigh" study. On day 10 postpartum, pups were removed from dam for 4 hours. The pups (as a litter)were weighed, given back to the dam and allowed to suckle for 30 minutes, then weighed again.
B, Pups were individually weighed every day until weaning on day 21.

SUMMARY

- hPRL is expressed in a human-like pattern in hPRL-BAC transgenic mice.
- Pit-hPRL is secreted and regulated by DA & E_2, both *in vitro* and *in vivo*.
- hPRL rescues the reproductive defects of the mPRL knock-out female mouse.
- hPRL increases in the spleen in response to immune challenge.

SIGNIFICANCE

These hPRL-BAC mice are currently being bred onto the NOD/SCID immunodeficient background to enable acceptance of human breast cancer xenografts. This will enable the direct study of prolactin actions in human breast cancer growth and metastasis, and in the interaction of cancer and inflammation.

These novel mouse models will enable, for the first time, *comprehensive* preclinical study of human breast cancer since they will produce all of the principal hormones that direct human mammary gland growth and differentiation.

This work was supported by DOD BC095713 to K.A.G.

Biological Characterization of Mice Expressing the Human Prolactin Gene.

H. R. Christensen[2], N.D. Horseman[2], K. A. Gregerson[1,2]

[1]James L. Winkel College of Pharmacy and [2]Dept. of Molecular and Cellular Physiology, University of Cincinnati, Cincinnati, OH 45267

INTRODUCTION

PRL is secreted by lactotrophs of the anterior pituitary into the general circulation. In humans and other primates, PRL is also synthesized and secreted by cells in "extrapituitary" tissues where it is believed to exert local, or paracrine, actions[1]. Several of these extrapituitary-PRL tissues serve a reproductive function (e.g., mammary gland, decidua, prostate, etc.), consistent with the hypothesis that local PRL production may be involved in (or, perhaps, required for) normal reproductive physiology. Expression of extrapituitary PRL is driven by the activity of an alternative promoter, 5.8 kb upstream of the pituitary transcription start site in the human PRL gene, which is absent from the rodent PRL gene[2].

RATIONALE

Knowledge of the physiological regulation and function of extrapituitary PRL is limited by the difference in rodent models and the relative inaccessibility of human experimental data. In addition, rodent PRL is essentially inactive on the human PRL receptor[3]. As a consequence, experimental transplantation of human cells and tissues into immunocompromised rodents are effectively PRL-deficient. To overcome these limitations we used a bacterial artificial chromosome (BAC) containing the entire human PRL (hPRL) gene[4] to generate mice that have been "humanized" with regard to the structural gene, regulatory sequenes, tissue expression, and secretory patterns of PRL. Here, we present characterizations of these animals, demonstrating that the human PRL (hPRL) transgene in the AP and spleen is responsive to known physiological regulators.

REFERENCES

[1] Gellersen, B., *et al.* 1994. *Mol Endocrinol 8*: 356-373.
[2] Berwaer, M., *et al.* 1994. *Mol Endocrinol 8:* 635-642.
[3] Utama, F.E., *et al.* 2006. *J Endocrinol 188:* 589.
[4] Semprini, S., *et al.* 2009. *Mol Endocrinol 23*: 529-538.

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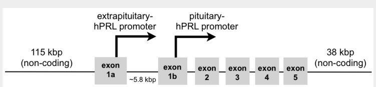
SPECIFIC AIMS

- Generate transgenic mice that express hPRL in a human pattern (in both pituitary and extrapituitary tissues).
- Demonstrate that the hPRL peptide is synthesized and secreted in the mice and regulated in a physiological fashion.
- Cross the hPRL-BAC mice onto the mPRL$^{-/-}$ background and determine if the hPRL rescues the reproductive phenotypes (infertility; alactation) of mPRL$^{-/-}$ females.

METHODS

Transgenic mice: The RP11-273G3 BAC was purchased from Children's Hospital Oakland Research Institute. This BAC is part of human chromosome 6 and contains the entire hPRL gene plus 115 kbp upstream and 38 kbp downstream of the gene locus (*see below*). Purified BAC DNA was provided to the Animal Models Core at Univ. of North Carolina for pronuclear microinjection into mouse oocytes.



Genotyping: Mice carrying the transgene were identified by PCR of genomic DNA identifying unique segments of the BAC at the 5' and 3' ends as well as within the hPRL coding region.

mRNA expression: Whole tissue samples were dissected from genotype-positive BAC mice, flash frozen in liquid nitrogen and stored at -80C until analyzed. Frozen tissues were homogenized in TRIzol and RNA extracted using standard protocol. Total RNA was reverse-transcribed and the cDNA used as the template for PCR using *primers specific to mPRL or hPRL*.

PRL measurement: Serum, culture medium, and tissue homogenates were assayed for PRL content using double-antibody RIA with reagents from Dr. A.F. Parlow. Primary antisera were specific for either hPRL or mPRL.

Anterior pituitary (AP) cell cultures: AP glands were rapidly dissected and cells dissociated in 0.2% trypsin for 15 min at 37°C. Following washes in Hank's buffer, cells were plated in 12-well dishes and cultured in DMEM + 10% horse serum overnight or for 6 days.

Animal studies: To test hPRL responsiveness, BAC-hPRL mice were subjected to various treatments and physiological challenges. (*See figure legends for details.*) BAC-hPRL mice were cross bred with mPRL-/- mice to produce mice expressing *only* hPRL.

RESULTS

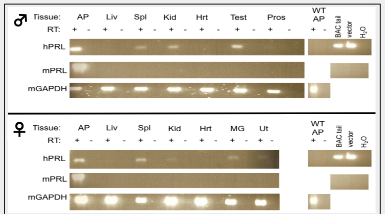
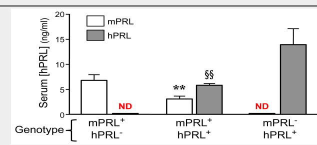


FIGURE 1: hPRL mRNA is expressed in human-like pattern in ♂ and ♀ hPRL-BAC mice.

RNA isolated from various tissues was analyzed by RT-PCR. mPRL transcript was detected in AP only, while hPRL was detected in AP, spleen (Spl), Kidney (Kid), mammary gland (MG), uterus (Ut), testis (Test) and prostate (Pros). *Note specificity of primers in WT AP & BAC vector.*



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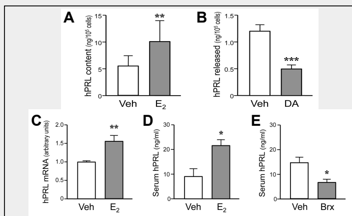

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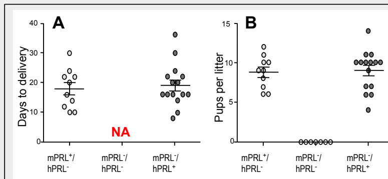
<u>*In vitro studies.*</u> Primary cultures of pituitary cells from ♀ hPRL-BAC mice were treated: **A.** 6 days with E_2 (80pg/ml) or vehicle, then cells collected; or **B.** 60 minutes with DA (100 nM), then medium collected. <u>*In vivo studies.*</u> Female hPRL-BAC mice were given: **C** and **D.** s.c. pellets of E_2 or vehicle, and killed14 days later (AP and serum collected); or **E.** s.c. injection of the DA agonist, bromocriptine (Brx), killed 90 min later and serum collected. Sera, cells and media were assayed for hPRL in RIA. AP hPRL mRNA was measured by qRT-PCR. *p<0.05, **p<0.01, ***p<0.001 *vs* vehicle control.



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A, Female mice were housed with proven sires and the day of vaginal plug was counted as day 0. *Note: No mPRL$^{-/-}$ mice without hPRL had litters.*
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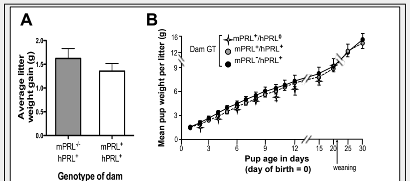

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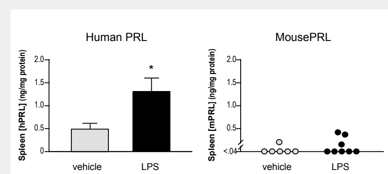

FIGURE 6: Immune challenge tends to increase hPRL, but not mPRL, in spleen.

Mice carrying both the endogenous mPRL gene and the hPRL-BAC transgene were injected with LPS (3 mg/kg; i.p.) and killed 18 h later. PRL levels in serum and spleen homogenates were measured by RIA. n = 6-8. "<0.04" indicates below assay detectability.

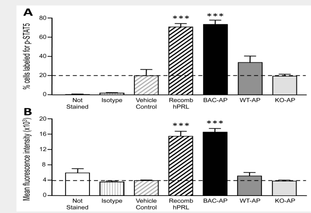

FIGURE 7: hPRL produced by transgenic mice is biologically active at hPRL-R..

Stat5 phosphorylation in human breast cancer T47D cells was measure by FACS following various treatments.

SUMMARY

- hPRL is expressed in a human-like pattern in hPRL-BAC transgenic mice.
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Completely Humanizing Prolactin Rescues Infertility in Prolactin Knockout Mice and Leads to Human Prolactin Expression in Numerous Extrapituitary Mouse Tissues

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ABSTRACT

A variety of fundamental differences have evolved in the physiology of the human and rodent prolactin (PRL) systems. Disruption (knockout) of either the PRL gene or its receptor (PRL-R) causes profound female reproductive defects at several levels (ovaries, pre-implantation endometrium, mammary glands). The PRL gene in humans and other primates contains an alternative promoter, 5.8 kb upstream of the pituitary transcription start site, that drives expression of PRL in "extrapituitary" tissues where PRL is believed to exert local, or paracrine, actions. Several of these extrapituitary-PRL tissues serve a reproductive function (e.g., mammary gland, decidua, prostate, etc.), consistent with the hypothesis that local PRL production may be involved in, and required for, normal reproductive physiology in primates. Rodent research models have generated significant findings regarding the role of PRL in reproduction, but the rodent PRL gene differs significantly from the human, most notably lacking the alternative promoter. Understanding of the physiological regulation and function of extrapituitary PRL has been limited by the absence of a readily accessible experimental model, since the rodent PRL gene does not contain the alternative promoter. To overcome these limitations, we have generated mice that have been "humanized" with regard to the structural gene and tissue expression of PRL. Here, we present the characterization of these animals, demonstrating that the human PRL (hPRL) transgene is responsive to known physiological regulators both *in vitro* and *in vivo*. More importantly, the expression of the hPRL transgene is able to rescue the reproductive defects observed in mouse PRL knock-out ($mPRL^{-}$) females, validating their usefulness in studying the function or regulation of this hormone in a manner that is relevant to human physiology.

INTRODUCTION

Prolactin (PRL) was discovered in the anterior pituitary gland (AP) as a lactogenic endocrine hormone (Riddle 1933; French article, 1928? Stricker and Greuder). Subsequent studies, culminating with targeted gene knockouts of mouse PRL (mPRL) and its receptor (mPRLR), have demonstrated that PRL has multiple essential roles in female fertility (Horseman 1997; Ormandy, 1997a; Ormandy 1997b; Lucas 1998; Bao 2007; Bachelot & Binart 2007).

PRL and growth hormone (GH) diverged from a common ancestral gene early in vertebrate evolution (Cooke & Liebhaber). In mammals, the PRL gene has undergone amplification and diversification at least twice (in rodents and ruminants), whereas in primates there is a single PRL gene and an amplified GH locus (Cooke & Liebhaber). In spite of the apparent simplicity of having a single PRL gene, primates have diversified the physiological potential of PRL by having evolved two distinct promoters that differentially regulate its expression (Berwaer 1994; Gellersen 1994). Understanding the human PRL locus has been hampered by the limited range of experimentally tractable models.

Physiological PRL synthesis and secretion in the pituitary gland is regulated primarily by hypothalamic dopamine (DA) and ovarian estradiol (E_2), which inhibit and stimulate PRL, respectively. DA is synthesized in cell bodies in the hypothalamic arcuate nucleus and released from terminals of the tuberoinfundibular dopamine neurons in the median eminence and pituitary stalk. Type 2 DA receptors (D2) on lactotrophs inhibit PRL synthesis and secretion through multiple signal transduction mediators. Estradiol acts via estrogen receptor-alpha (ERα) to induce PRL gene and protein expression. There are a variety of additional PRL regulating factors that play subsidiary physiological roles, but can be very important in disease states (Horseman & Gregerson 2010).

Expression of PRL in extrapituitary tissues is well documented, but poorly understood (Ben-Jonathan 1996; Featherstone 2012). In primates, including humans, extrapituitary PRL is driven by a distal promoter that is upstream of an extra non-coding exon (exon 1a) (REFS). Although rodents do not have a distal extrapituitary promoter, PRL has been shown to be expressed in lymphocytes, uterus, and mammary glands (REFS). Potential functions of extrapituitary PRL *in vivo* have been demonstrated using PRL knockout mice (REFS). In the uterus of mice, local PRL suppresses expression of genes that are detrimental to pregnancy (REF, BAO et al). In mouse mammary glands, local PRL is induced by AKT signaling, and drives proliferation in the peripartum (REFS).

In primates, the extrapituitary PRL promoter drives expression in a larger variety of tissues, but ascribing functions to human extrapituitary PRL *in vivo* has been difficult. Among the tissues that express PRL in humans are several male and female reproductive organs such as the prostate, uterus, and mammary glands (Escalada J 1997; Iwasaka 2000; Lkhider 1996; Tseng 1999; Nevalainen 1997; Golander 1978; Brown 1994; Kurtz 1993). The mature peptide in these tissues is identical to that produced in the AP.

Another fundamental difference between rodent and human PRL is that female reproduction in laboratory rodents is supported by a complex pattern of twice-daily PRL surges that are stimulated by copulation. Absent the long luteal phase of the human menstrual cycle, the short rodent estrous cycle is suspended by the PRL surges, providing sufficient time for implantation and development of a hormonally active placenta (REFS).

To address the multiple differences between the rodent and human PRL systems *in vivo*, we have developed mice in which the PRL is replaced by the PRL locus from the human genome (hPRL), including both the proximal and distal promoters. These mice reveal that the reproductive deficits are rescued by hPRL, and that hPRL is expressed in a variety of tissues that do not express measurable mPRL.

METHODS

Animals and Reagents

Male and female C57Bl/6J mice purchased from Jackson Laboratories (Bar Harbor, ME) were used to establish breeding colonies with transgenic founder animals. Animals were maintained on a 14-hour light / 10-hour dark cycle (lights on at 06:00am) with food and water available *ad libitum*. Staging of estrous cycle in female mice was based on vaginal cytology obtained by daily vaginal lavage. In all studies requiring determinations of circulating PRL levels, animals were sacrificed by rapid decapitation following 5 days of acclimation to the guillotine in order to obtain "unstressed" levels of serum PRL. Trunk blood was collected and allowed to clot overnight at 4ºC. Serum was separated by centrifugation at 10,000 x g for 5 minutes (4ºC) and stored at -20ºC until analyzed for hPRL and mPRL protein. All animal handling and procedures were carried out in a facility accredited by the American Association for the Accreditation of Laboratory Animal Care and were approved by the University of Cincinnati's Institutional Animal Care and Use Committee.

Horse serum and fetal bovine serum were purchased from HyClone (Thermo Scientific, Logan, UT) and Atlas Biologicals, Inc. (Fort Collins, CO), respectively. RPMI 1640 medium, L-glutamine, and penicillin-streptomycin were from Biofluids, Inc. (Rockville, MD). For RIA of mPRL and hPRL, antibodies and purified hormones were purchased from Dr. A. Parlow through the National Hormone and Peptide Program (UCLA). Normal rabbit serum (NRS) and anti-rabbit gamma globulin (ARγG-P_4) were purchased from Antibodies, Inc. (Davis, CA). For the

flow cytometry, Alexa Fluor® 647-conjugated mouse anti-Stat5(pY694) and mouse IgG2a (κ isotype control) for flow cytometry were purchased from BD Biosciences (San Jose, CA). Molecular biology reagents used included: QuantiTect® reverse transcription and QuantiFast® SYBR Green PCR kits from QIAgen (Valencia, CA); AccuPrime™ Taq PCR kits from Invitrogen (Carlsbad, CA); FastStart® PCR reagents from Roche (Indianapolis, IN), and primers from Integrated DNA Technologies (San Diego, CA). Placebo and 17β-estradiol pellets were obtained from Innovative Research of America (Sarasota, FL). All other reagents and culture media, unless otherwise noted in specific methods below, were purchased from Sigma Chemicals (St. Louis, MO).

Generation of hPRL-expressing mice

The BAC RP11-273G3 construct was purchased from the Children's Hospital Oakland Research Institute (CHORI, http://www.chori.org/) as an LB agar stab. This construct contains the entire hPRL gene, including the pituitary and extrapituitary promoters as well as 115 kb upstream and 38 kb downstream of the gene-encoding sequences. This sample was streaked onto LB agar plates containing 12.5 µg/mL chloramphenicol to isolate single bacterial colonies, and one was selected for growth in LB broth containing 12.5 µg/mL chloramphenicol. DNA was isolated using the QIAgen EndoFree® plasmid kit, and the purified DNA was submitted to the Animal Models Core at the University of North Carolina at Chapel Hill School of Medicine (http://www.med.unc.edu/amc) for pronuclear microinjection into fertilized C57BL/6J X DBA2 hybrid mouse oocytes and implantation into pseudopregnant dams. Resulting offspring positive for the transgene ($hPRL^+$) were shipped to the University of Cincinnati where they were crossed onto the C57BL/6J background. F2 generation mice were then crossed onto with C57Bl/6J mice in which the mouse PRL gene was deleted (mPRL^{-} Horseman *et al.*, 1997).

Genotyping

Founder animals and offspring carrying the BAC transgene were identified by PCR analysis of genomic DNA isolated from tail biopsies using an ethanol extraction protocol (Wang and Storm, 2006). Presence of the full BAC construct was determined using primers (Table 1) designed to recognize unique sequences in the non-coding regions at the 5' and the 3' end of the BAC construct (GenBank ID AQ485155.1 and AQ485157.1, respectively; www.ncbi.nlm.nih.gov). The gene of interest (hPRL; accession number NM_000948) was also verified using hPRL-specific primers designed against sequences in exon 1 (Supplementary Table 1). The amplification protocols utilized an initial hot start at 94°C (2 minutes) followed by 30 sequential cycles of the following temperatures (45-second duration each): 94°C (denaturing); Tm°C (annealing; see Table 1); and 72°C (extending). All reactions were incubated at 72°C for 5 min after the last cycle for final extension, then kept at 4ºC until analyzed by agarose gel (1.2%) electrophoresis. For animals bred onto the mPRL^{-} background, the mPRL genotype was determined using primers designed to recognize the wild-type (WT, mPRL^{+}) allele or the knockout (KO) allele containing the NEO cassette (Supplementary Table 1).

The nomenclature used within for the animals includes designations for the human (h) and mouse (m) PRL genes and the allelic genotypes according to the following scheme: hPRL^{+} = hPRL$^{+/0}$; hPRL^{0} = hPRL$^{0/0}$; mPRL^{+} = mPRL$^{+/?}$; mPRL^{-} = mPRL$^{-/-}$.

Anterior pituitary cell dissociation

Anterior pituitary cells were dissociated as previously described (Gregerson *et al.*, 2001). Briefly, AP glands (posterior pituitary removed) were minced and enzymatically dissociated in Hank's Balanced Salt Solution (with Ca^{2+} and Mg^{2+}) containing 0.20% trypsin for 15 minutes at 37°C. The digested tissue was washed in Hank's Ca^{2+}, Mg^{2+}-free medium (Hank's CMF) and triturated in Hank's CMF containing deoxyribonuclease-I (0.075 mg/mL) and trypsin inhibitor (3.75 µg/mL). The final cell suspension was filtered through a sterile, 20-µm pore nylon mesh. Cell yield was quantitated using a hemacytometer, and viability was determined to always be in excess of 96% based on trypan blue exclusion.

In vitro studies on dissociated AP cells

Cells dispersed from AP glands of males or females (random-cycling unless otherwise noted) were plated onto sterile, poly-L-lysine-treated glass coverslips ($5x10^5$ cells/slip) and maintained in multi-well dishes in culture medium (DMEM with 10% heat-inactivated horse serum; "DMES") at 37°C in 5% CO_2. Experiments assessing acute PRL release were performed 2 days after dissociation. Cells were pre-incubated in a standard external solution (SES; in mM: 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES-HCl; 37°C, 5% CO_2) containing 0.01% BSA (SES-BSA). Following 30 minutes of pre-incubation, coverslips were moved to fresh wells containing 1 ml of SES-BSA with one of the following treatments: vehicle (0.5 mM ascorbic acid), DA (300 nM), or thyrotropin-releasing hormone (TRH, 100 nM). At the end of a 1-hour incubation the treatment media was collected, spun at 1,000 rpm to pellet any unplated cells (5 minutes; 4ºC), and the supernatant stored at -20ºC until analyzed for hPRL and mPRL protein. DA and TRH solutions were made fresh immediately before each release experiment.

To assess PRL synthesis/storage *in vitro*, dissociated AP cells were plated directly into the wells of 12-well culture plates. Cells were incubated at 37°C in a DME modified medium base (no phenol red, 25 mM Hepes-HCl, 10 µg/mL insulin, 15% charcoal-stripped, heat-inactivated horse serum, 2.5% charcoal-stripped, heat-inactivated fetal bovine serum, 40 µg/mL gentamycin sulfate) containing vehicle (0.003% ethanol), 17ß-estradiol (E_2, 100 pM) or the estrogen receptor antagonist ICI 182,780 (ICI, 10 nM) for 6 days (media changed on day 3). On the final day, media was removed and cell lysates collected by triturating each well with 200µL dH_2O. Following trituration, 200µL of 1.8% saline was added to each sample to normalize tonicity, the lysates were spun (1,000 rpm; 5 min) and the supernatants were frozen at -20ºC until analyzed for hPRL and mPRL protein.

In vivo studies

To assess pituitary PRL responsiveness to estrogen, intact male and female mice were implanted with subcutaneous pellets of 17ß-estradiol (E_2, 0.05 mg total dose, 21-day release) or placebo, and sacrificed 14 days later. The AP glands were dissected, weighed (wet weight; posterior pituitary gland removed) and bisected. One half of each gland was flash frozen in liquid nitrogen and stored at -80ºC until analyzed for mRNA. The second half of each gland was homogenized in ice-cold dH_2O using a ground glass micro-homogenizer. An equal volume of 1.8% saline was added to normalize tonicity, and the sample was spun at 12,000 x g for 10 minutes (4ºC). Both the supernatant and cell pellet were frozen separately at -20ºC until analyzed for PRL protein or total protein, respectively. To assess pituitary PRL responsiveness to DA, intact female mice received a subcutaneous injection of either vehicle or the D_2R agonist

bromocriptine (Brx; 2.5 mg/kg), between 09:00 and 11:00am on diestrus and were sacrificed 90 minutes later.

Some animals were subjected to an immune challenge by intraperitoneal injection of lipopolysaccharide from *Escherichia coli* (LPS; 3 mg/kg) and sacrificed 16 hours later. In addition to serum, several whole organs were harvested, including spleen, thymus and anterior pituitary. Each was weighed, immediately homogenized in ice-cold dH_2O, and processed as described above.

To assess sexual maturation and fertility, female mice were monitored for vaginal opening and then daily smears taken to assess estrous cyclicity. Beginning at 6 weeks of age, proestrous females were housed with proven sires, and the time to delivery (days) and litter sizes were recorded. As an index of the lactation performance of each dam, pups were weighed daily for the first 12 days of postnatal life, and on 15, 20, 25 and 30 days of age (weaning on day 21). Lactation was also assessed in "weigh-suckle-weigh" experiments (WSW). For WSW, on postpartum day 10 (day 0 = day of delivery) pups were separated from the dam for a period of 4 hours. Six pups were then weighed individually, returned to the dam, and allowed to suckle for 30 minutes, then weighed again. The pup weight gain (post- minus pre-suckling weight) was calculated as a measure of milk production. At the end of the 30-minute suckling period, a blood sample from each dam was rapidly collected by tail snip, and serum harvested as described above for assessment of suckling-induced PRL release.

Analysis of mRNA expression

Whole tissue samples [mouse AP, hypothalamus (HTH), heart (HRT), kidney (KID), liver (LIV), spleen (SPL), mammary gland (MG), uterus (UT), ovaries (OV), testes (TEST), and prostate (PROS)] were dissected from genotype-positive BAC transgenic mice, flash frozen in liquid nitrogen, and stored at –80°C. Frozen tissues were homogenized in ice-cold TRIzol, and RNA was extracted according to standard protocol guidelines (Invitrogen, Cat #15596-018). Reverse transcription (RT+) of 0.5µg total RNA was completed in a final volume of 10µL following manufacturer's protocol (QIAgen, Cat #205310). Negative control reactions (RT-) were handled in an identical manner, except that reverse transcriptase enzyme was omitted. Primers corresponding to regions specific for either mPRL or hPRL were designed (Supplementary Table 2) and used in PCR with the cDNAs from the above tissues as templates. Each PCR reaction was carried out in a final reaction volume of 12.5 µL containing 1 µL of a RT reaction solution and reagents from Roche FastStart PCR). Following an initial hot start at 94°C for 2 minutes, reactions were sequentially cycled 35 times for 45-second durations at each of the following temperatures: 94°C (denaturing); Tm°C (annealing; *see* Table 1); and 72°C (extending). All reactions were incubated at 72°C for 5 minutes after the last cycle for final extension, and then stored at 4°C until analyzed by gel electrophoresis.

Immunoassays of PRL protein

Mouse PRL and hPRL proteins in serum, tissue homogenates and cell lysate samples were determined by homologous double-antibody RIAs using species-specific antibodies. Antibodies were diluted in 0.25% NRS and used at final concentrations of 1:300,000 for α-hPRL-3 (AFP-C11580) and 1:450,000 for α-mPRL (AFP-131078). These two RIAs exhibited no measureable cross-reactivity with the PRL from the other species (see Supplementary Figure S1), which was essential to these experiments. This was not the case for Western blots, in which mPRL could be detected by the α-hPRL antibody (several were tested).

pSTAT5 in T47D cells

Human breast cancer T47D cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin (50 IU/mL and 50 µg/mL respectively). For STAT5 activation experiments, T47D cells were seeded into T25 flasks (~250,000 cells/flask) and allowed to plate for 8-9 hours. Medium was then replaced with RPMI 1640 containing 0.1% BSA for serum deprivation. Sixteen hours later the medium from each flask was replaced with a treatment medium and incubated at 37ºC for 20 minutes. Treatments included the following: control (vehicle), recombinant hPRL (10 nM), extract from BAC APs (to make 10 nM hPRL, as determined by RIA) and the same dilution of extract from PRL-/- APs. At the end of the 20 minutes the treatment medium was removed and cells were trypsinized for 2 minutes (0.05% trypsin, 2mM EDTA in PBS; 37ºC) then triturated to suspend single cells. Immediately, ice-cold 4% paraformaldehyde (in PBS) was added to a final concentration of 2%, and the T47D cells were stored at 4ºC overnight.

The next day, cells were washed once with ice-cold PBS then resuspended in 1 mL of ice-cold 100% methanol to permeabilize the cell membranes.

Accuri Cytometer C6 Flow Cytometer

Statistics

One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test were used for statistical analysis of the *in vitro* PRL release data, the fertility parameters (vaginal opening, time to delivery, litter size), and the flow cytometry data. Student's *t*-test was used to compare treatment groups in the *in vivo* studies using E_2, Brx, and LPS treatments. Student's t-test was also performed on the individual and litter weight gain assessed in the WSW experiments.

RESULTS

hPRL rescues fertility in mPRL⁻ females

Given the profound and multifaceted fertility defects in female PRL knockout mice, we had a primary interest in determining whether the hPRL transgene, driven by human regulatory regions, would rescue these reproductive deficits.

As previously described (horseman 1997), vaginal opening (VO) was delayed in $mPRL^-$ females in comparison with their normal ($mPRL^+$) controls ($p < 0.001$; Figure 5A); and $mPRL^-$ estrous cycles were irregular, often having multiple days of proestrus and/or estrus. Mice expressing the hPRL transgene along with mPRL ($hPRL^+$:$mPRL^+$) were indistinguishable from the normal $mPRL^+$ controls. The hPRL transgene alone ($hPRL^+$:$mPRL^-$) restored the time to VO to control levels (Figure 5A), and these $hPRL^+$:$mPRL^-$ females had regular estrous cycles with single days of proestrus and estrus. Thus, hPRL restored the timing of puberty and reproductive cycling to normal patterns.

Mating units were assembled in which proven sires were paired with proestrous dams of each of the following types: (1) $hPRL^0$:$mPRL^+$, (2) $hPRL^+$:$mPRL^+$, (3) $hPRL^0$:$mPRL^-$, and (4) $hPRL^+$:$mPRL^-$. As expected, after several consecutive months the $mPRL^-$ females were unable to produce any litters. Dams expressing only hPRL (group 4, $hPRL^+$:$mPRL^-$) produced litters at the same rates (Figure 5B) and of similar sizes (Figure 5C) compared with either normal controls ($hPRL^0$:$mPRL^+$) or bigenic controls ($hPRL^+$:$mPRL^+$). These data demonstrate that the hPRL structural gene, controlled by hPRL regulatory elements, rescued the full range of female reproductive deficits during puberty, the ovarian cycle, embryonic development, and late gestation in the absence (or presence) of a biologically active mouse PRL gene.

Mammary Development and Lactation in hPRL mice.

MG development in PRLKO females was arrested in pubertal state, in which the epithelium consisted of a simply branched ductal tree with persistent terminal endbuds (Horseman et al 97, Figure XXXX). The hPRL transgene restored normal MG development in nulliparous (virgin) females, such that the glands differentiated fully into complex epithelial networks of ducts decorated by side branches and alveolar buds, and without persistent terminal endbuds (Figure XXX). Development in the $hPRL^+$; $mPRL^-$ females was indistinguishable from the normal and bigenic controls (Figure XXXX and XXXX).

Lactational competence of hPRL females was measured acutely using a weigh-suckle-weigh (WSW) protocol. Pups were allowed to nurse after a 4-hour fast. The mothers expressing only hPRL provided identical amounts of milk to their pups during a 30-minu suckling period, compared with control animals ($hPRL^+$; $mPRL^+$) (Figure XXXA). The dams from both groups demonstrated increased circulating hPRL protein levels after a 30 min bout of nursing (Table XXX?).

Lactational competence over the course of the full lactation cycle was assessed by monitoring the growth rates of pups born to mothers of hPRL transgenic mice. The rate and extent of growth of pups born to $hPRL^+$ ($mPRL^-$) mothers was indistinguishable from that of pups born to wildtype control ($hPRL^0$:$mPRL^+$), or bigenic control mothers ($hPRL^+$; $mPRL^+$) (Figure XXXB).

Female reproduction in $hPRL^+$; $mPRL^-$ female mice (*i.e.*, humanized PRL) was indistinguishable from normal from the onset of puberty through gestation and lactation, despite the fundamental differences in the organization and regulation of the human and rodent PRL genes. Additional experiments were done to test specific regulatory mechanisms of hPRL gene expression in the mouse.

Humanized PRL is responsive to E_2

Regulation of hPRL *in vivo* was studied by continuous E_2 treatment for 14 days via implanted pellets, which had the expected hypertrophic effect on the AP glands of both male and female $hPRL^+$ mice (>50%, Figure XXXX). Gene expression of hPRL was induced by *in vivo* E_2-treatment in pituitaries of both males and females (Figure XXX). Basal levels of serum hPRL were significantly lower in male mice compared with females, and E_2 increased circulating hPRL by approximately 2-fold in both males and females (Figure XXX).

To analyze direct E_2 actions on the pituitary cells, primary cultures of dissociated AP gland cells were cultured for six days in a hormone-depleted medium (see Methods). Cells were derived from mice that were bigenic for PRL ($hPRL^+$;$mPRL^+$), and expression of both genes and proteins were separately measured. Control cells were cultured in medium with hormone diluent (0.0003% final [ethanol]), and treatment groups included either E_2 (100 pM), or ICI (10 nM). Significant increases in hPRL and mPRL protein contents of AP cells were observed following 6 days of E_2 treatment of cells collected from either males or females (Figure XXX). The decrease of PRL by ICI treatment of cells from females was significant for hPRL ($p<0.01$), but not for mPRL.

Humanized PRL secretion is regulated by dopamine

DA is the predominant acute physiological regulator of PRL secretion (REF Horseman and Gregerson). Brx (a D2 receptor agonist) injection suppressed serum PRL levels of both hPRL and mPRL in female mice (Figure XXX). Correspondingly, DA inhibited secretion of both hPRL and mPRL during a 1-hour *in vitro* release assay (Figure XXXX).

The hPRL gene is expressed in numerous extrapituitary mouse tissues

Based on previous studies of extrapituitary PRL expression (REFS), a variety of tissues were harvested from bigenic $hPRL^+$;$mPRL^+$ mice and used to determine the expression patterns of hPRL and mPRL transcript simultaneously. Assays were performed on males and nulliparous (*i.e.*, virgin) female mice. Both mPRL and hPRL were expressed in the AP glands (Figure XXX). In contrast, hPRL, but not mPRL, expression was detected in several extrapituitary sites, including the spleen, kidney, and reproductive tissues of both males and females (MG, UT, PROS, and TEST; Figure XXX).

LPS induces human, but not mouse, PRL expression in the spleen

To address the regulation of humanized PRL in immune-related cells of mice we used LPS treatment (REFS). Serum levels of both hPRL and mPRL were elevated by LPS treatment of $hPRL^+$;$mPRL^+$ mice (Figure XXX), consistent with a non-specific stress reaction to systemic LPS. Pituitary contents of both forms of PRL were lower, albeit not significantly, in LPS-treated mice, consistent with release of a portion of the stored pools of these hormones. In the spleens, hPRL content was significantly elevated in response to LPS treatment, whereas mPRL content was generally below the detection limit, and not stimulated by LPS.

Mouse-derived hPRL activates STAT5A in human breast cancer cells

Given that rodent PRL is a poor agonist for human PRL receptors (REFS), it was important to establish whether mouse-expressed hPRL retained the ability to activate PRL receptors in human cells. To this end, human breast cancer T47D cells were treated with protein extracts from either $hPRL^{+}$;$mPRL^{-}$ or $hPRL^{-}$;$mPRL^{+}$ (WT) mice. To estimate the potency of mouse-expressed hPRL, we used recombinant hPRL (10 nM), and the concentrations of hPRL or mPRL in the pituitary extracts (measured by RIA) were adjusted so that the treatment doses were also 10 nM. STAT5 activation was measured by immunostaining and flow cytometry.

As shown in Figure XXX (and Supplement YYY), hPRL extracted from AP glands of transgenic humanized mice activated STAT5A signaling (phospho-STAT5A) in T47D cells. In contrast, extracts from either wildtype ($mPRL^{+}$) or knockout ($mPRL^{-}$) pituitary glands were inactive in T47D cells. The similar STAT5A activation by recombinant hPRL and mouse-derived hPRL indicates that their potencies are roughly equivalent.

DISCUSSION

There are numerous and well-documented differences in the regulation and biological activities of primate (*e.g.,* human) and rodent (*e.g.,* mouse) PRL. With the ultimate intention of understanding the biology and pathology of human PRL, we were motivated to use molecular genetic approaches to engineer a new mouse model in which we inserted a large region of human DNA that includes both the structural gene and all of its known regulatory elements (REFS).

One well-established difference between the human and rodent PRL systems is that rodents are completely reliant on pituitary PRL secretion for female fertility (REFS). The survival and function of the rodent corpus luteum (CL) depends on pituitary PRL, whereas the human CL is not dependent on pituitary PRL (REFs). Elevated progesterone secretion from the CL induces functional receptivity in the uterus (REF), and promotes early embryo survival in the oviducts (REFS). Consequently, PRL and PRL-R knockout mice are completely infertile (REFS).

Our results show that replacing the mouse PRL gene with the human PRL transgene fully restored fertility and lactation in females. This result is unsurprising at a superficial level. However, the various patterns of PRL expression in female reproduction, from puberty through lactation, are the culmination of a complex chain of developmental and physiological events (REF, Carriere, Gleiberman, Lin, Rosenfeld 2004). The rescuing of mouse fertility by hPRL demonstrates that this chain of events can be replicated in a heterologous system.

The hPRL gene, in the mouse context, was induced by E_2, and was inhibited by Brx (a D2 agonist). These regulatory effects were seen both *in vivo*, where the hypothalamic control mechanisms are intact, and *in vitro*, showing that the effects of E_2 and Brx on the lactotrophs are direct. Since these two signals are the primary direct regulators of PRL during the reproductive cycle, one assumes that the estrogenic and dopaminergic control of hPRL accounts, in large measure, for the normalization of reproductive development, fertility, and lactation in these animals. There are other important PRL regulatory mechanisms, such as opioid and serotonergic signaling, which act indirectly at the hypothalamic level (REF), and may or may not be affected by hPRL.

Extrapituitary expression of hPRL. Although PRL expression outside the AP gland has been most extensively described in humans (REFS), species other than primates express extrapituitary PRL. In the mouse, extrapituitary PRL is expressed in the endometrial stroma, where it promotes fetal survival (REFs, Bao, et al.), and in the lactating mammary gland, where it stimulates epithelial expansion in the peripartum (REFs, Naylor, et al; Chen et al). However, the expression of extrapituitary PRL is not a consistent feature in mammals. Unlike mice and humans, rabbits, dogs and armadillos do not express PRL in the endometrium (REF, Emera, et al). The molecular evolution of extrapituitary PRL expression in mammals is complex, with multiple species (rodents, elephants, primates, and possibly others) having independently evolved genomic mechanisms to drive PRL expression in extrapituitary tissues (REF, Emera, et al). In primates, expression of extrapituitary PRL is a consequence of regulatory sequences derived from insertion of transposable elements (MER39/MER20) approximately 6 kb upstream of the structural gene (REF Gerlo, et al; Emera et al). The independent evolution of extrapituitary PRL expression in multiple phylogenetic clades of mammals strongly indicates that this system has been useful for adaptation to particular selective pressures, even though it is not essential for basic physiological functions. A related case-in-point is murine PRL-like protein A (PLP-A), which is expressed in the mouse placenta. Although it is not essential under conventional conditions, PLP-A is required for reproduction under the physiological stress of

reduced oxygen tension, such as occurs at high elevations (REF Ain R, Dai G, Dunmore JH, Godwin AR, Soares MJ.). This is an important example of how genes in the PRL family could evolve under selection pressure.

The convergent evolution of extrapituitary PRL expression in rodents and primates poses a significant challenge for understanding human PRL biology. Whereas extrapituitary PRL in mice appears to be expressed at biologically meaningful levels in lactating mammary gland and in the uterus during pregnancy (REFS), human PRL is expressed in a much larger variety of tissues and physiological, states Zinger M, McFarland M, Ben-Jonathan N. Phelps JY, Bugg EM, Shamblott MJ, Vlahos NP, Whelan J, Zacur HA OTHERS.

We studied the expression of extrapituitary hPRL under baseline conditions in males and in females that were non-pregnant and non-lactating. Among the tissues studied, hPRL was expressed in the spleen, kidney, mammary gland, uterus, prostate gland, and testes. Another human organ in which PRL has been documented is the brain (REF), but the complexity of studying brain PRL expression demanded that we defer consideration of the brain to future studies. In general, the tissues that express hPRL in the mouse correspond to those that have previously been documented to express PRL in the human (REFS, esp BenJon review). The apparent fidelity of hPRL expression in this mouse model is illustrated by its expression in the male testis, where hPRL mRNA was expressed robustly. Similarly, in human testis PRL is expressed at the mRNA level, and levels of hPRL protein in human testis were substantial (>50ng/g wet weight) (REF, G. Untergasser a, W. Kranewitter a, P. Schwa¨rzler a, S. Madersbacher c, S. Dirnhofer a,b,). We did not observe an obvious difference in male reproductive physiology in the hPRL mice under standard laboratory conditions. Therefore, it will be important to study testicular responses to physiological stresses in order to understand whether the substantial testicular hPRL expression has important roles in adaptation. These questions would be impossible to address adequately in humans, but can readily be studied in the humanized mouse model.

We studied female tissues only from virgin female mice. Because hPRL in female reproductive tissues is dynamically regulated by changing reproductive status, this new model provides avenues for future *in vivo* studies of extrapituitary hPRL in females.

Induction of hPRL by LPS. We also examined PRL expression in the spleen after a physiological challenge (LPS stimulation). In this experiment we used bigenic mice (wildtype for mPRL and transgenic for hPRL). Serum levels of both hPRL and mPRL were increased in response to LPS stimulation. However, because LPS elicits a non-specific systemic stress, the observed change of serum PRL likely includes a large component of secretion from the AP. Corresponding to the expression of mRNAs for mouse and human PRL mRNA, hPRL protein, but not mPRL, was detectable in unstimulated mouse spleens, and LPS stimulated greater than a 2-fold increase in spleen hPRL. In the mouse spleens, 4 of the 14 animals tested showed very low, but detectable mPRL protein, presumably derived from the blood, not local synthesis. The induction of splenic hPRL synthesis in the mouse is consistent with induction of reporter gene activity observed in transgenic rats in which EGFP expression is under the control of the human PRL promoter (Semprini S, Friedrichsen S, Harper CV, McNeilly JR, Adamson AD, Spiller DG, Kotelevtseva N, Brooker G, Brownstein DG, McNeilly AS, White MR, Davis JR, Mullins JJ).

Conclusions. Human PRL physiology is poorly understood at many levels because of evolutionary adaptations that have led to major differences in the biological activity and regulation of PRL in humans and typical rodent models. We have developed a mouse model that expresses hPRL under the control of its entire genomic context, and showed that hPRL rescues

the physiological defects caused by disrupting the mouse PRL gene. In this model, hPRL is expressed in tissues that do not typically express mPRL.

ACKNOWLEDGEMENTS

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Supplementary TABLE 1 Genotyping primers and annealing temperatures.

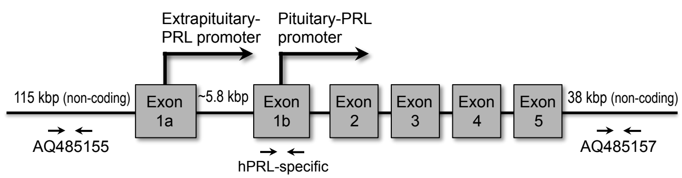
TARGET	PRIMER SEQUENCE	T_M
hPRL exon 1	forward 5' – TTT CTG GCC AGT ATG TCT TCC TGA – 3' backward 5' – GTC TCA CGG TTT TCT CTT TCC CAG – 3'	58°C
AQ485155	forward 5' – TGT GTG AGG TAC GGA AAC GA – 3' backward 5' – GCC ACT TGG GAA ATC AAG AG – 3'	54°C
AQ485157	forward 5' – AAA TTC TTG GCA AGG TAG CA – 3' backward 5' – CAT TCT TCT GGG GGA ACA AA – 3'	54°C
mPRL wild type	forward 5' – ATG GTG GAT TAG CCG GAA GT – 3' backward 5' – TTT CCA TGA GTC GGA AAA GC – 3'	54°C
mPRL knock-out (neo cassette)	forward 5' – ATT GCA TCG CAT TGT CTG AG – 3' backward 5' – TTT CCA TGA GTC GGA AAA GC – 3'	54°C

Supplementary TABLE 2 Primers and annealing temperatures used in RT-PCR.

TARGET	PRIMER SEQUENCE	T_M
hPRL	forward 5' – CAT GGA AAG GGT CCC TCC TG – 3' backward 5' – CGT ACT TCC GTG ACC AGA TG – 3'	55.5°C
mPRL	forward 5' – GGA GAA GTG TGT TCC CAG C – 3' backward 5' – CAG CGA ATG GTG TTG CGC – 3'	68°C

TABLE 1

Dam genotype	# Dams	# Litters	Avg litter size
$hPRL^+$; $mPRL^+$	6	11	10 ± 2.5
$hPRL^0$; $mPRL^-$	5	0	NA
$hPRL^+$; $mPRL^-$	7	15	8 ± 2.5



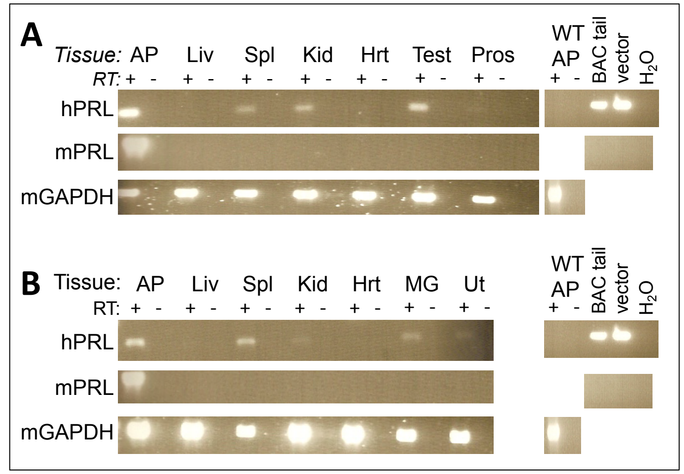


Figure 1. Human PRL transcript is expressed in a human-like pattern in BAC-transgenic mice. Tissues from genotype-positive female (**A**) and male (**B**) mice, on the mousePRL wild type background, were analyzed by RT-PCR. Both hPRL and mPRL transcript was present in the anterior pituitary gland (AP). Several non-pituitary tissues also expressed hPRL, but not mPRL, indicating the activation of the 1a-hPRL promoter in these tissues. For every tissue, a substantial product was present for mGAPDH, demonstrating the quality of mRNA, and no product was produced if RT enzyme was omitted (RT-) demonstrating the absence of genomic contamination. Panels on the right demonstrate species-specificity of the primers. Liv, liver; Spl, spleen; Kid, kidney; Hrt, heart; Test, testis; Pros, prostate; MG, mammary gland; Ut, uterus.

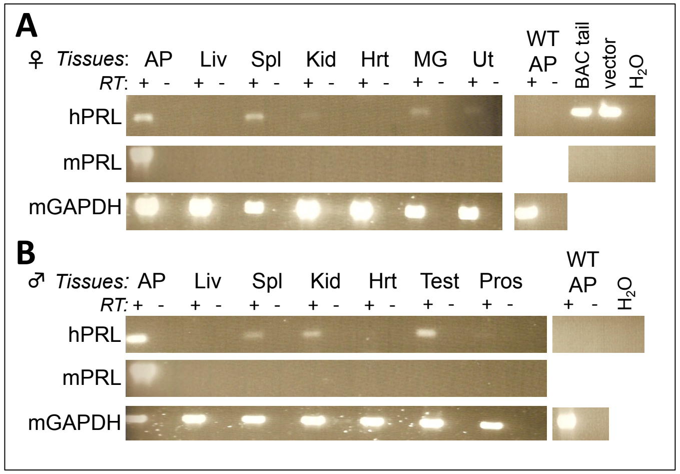
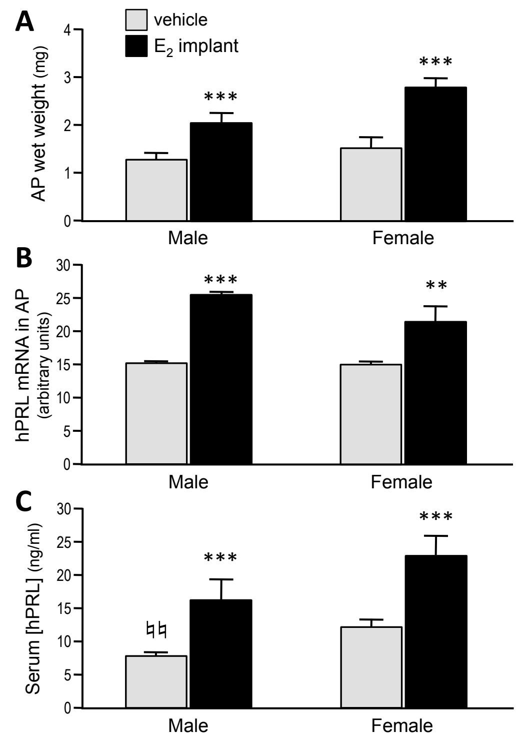
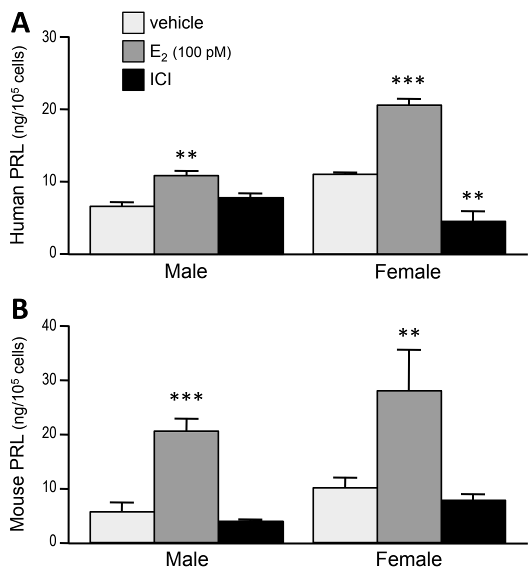
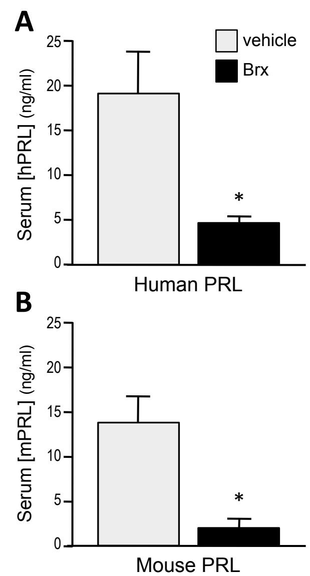
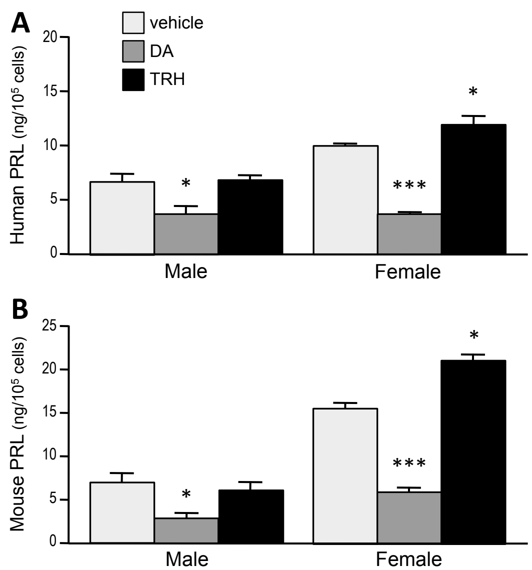


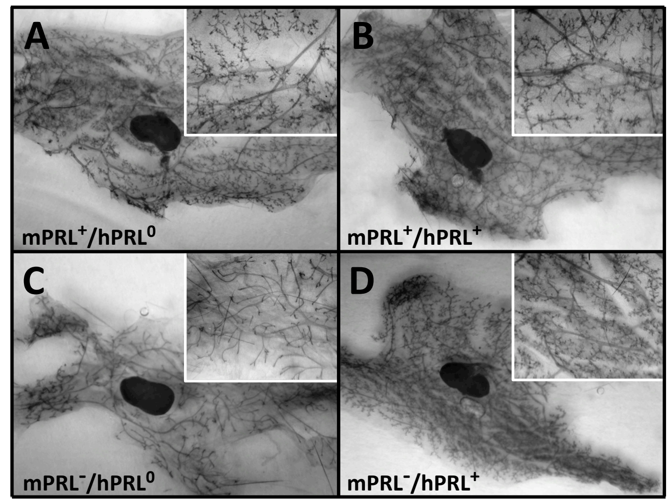
Figure 1. Human PRL transcript is expressed in a human-like pattern in BAC-transgenic mice. Tissues from genotype-positive female (**A**) and male (**B**) mice, on the mousePRL wild type background, were analyzed by RT-PCR. Both hPRL and mPRL transcript was present in the anterior pituitary gland (AP). Several non-pituitary tissues also expressed hPRL, but not mPRL, indicating the activation of the 1a-hPRL promoter in these tissues. For every tissue, a substantial product was present for mGAPDH, demonstrating the quality of mRNA, and no product was produced if RT enzyme was omitted (RT-) demonstrating the absence of genomic contamination. Panels on the right demonstrate species-specificity of the primers. Liv, liver; Spl, spleen; Kid, kidney; Hrt, heart; Test, testis; Pros, prostate; MG, mammary gland; Ut, uterus.

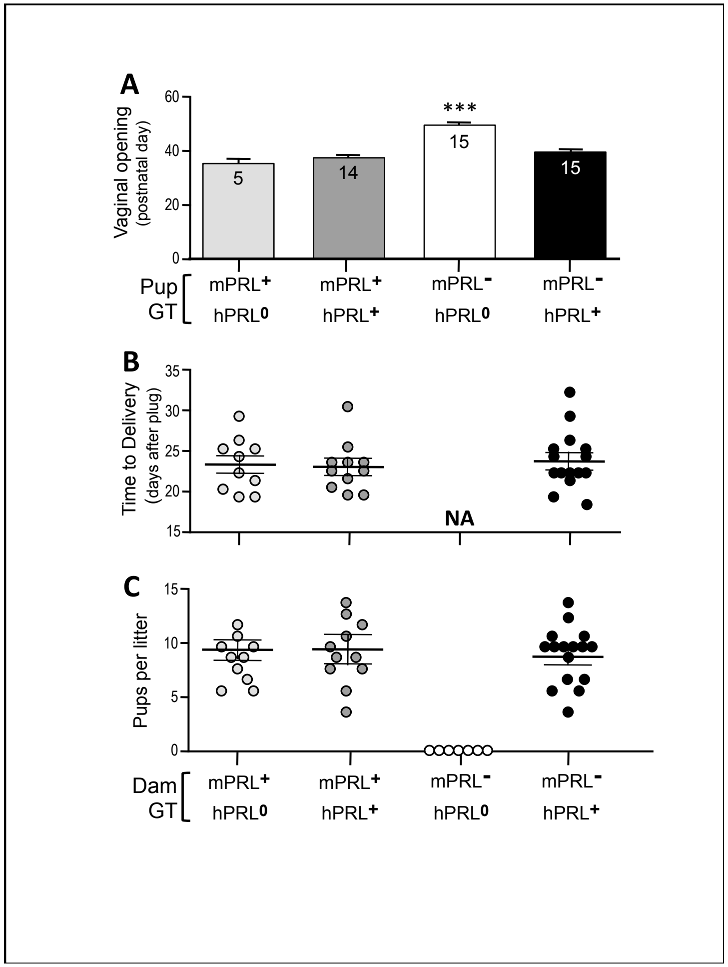


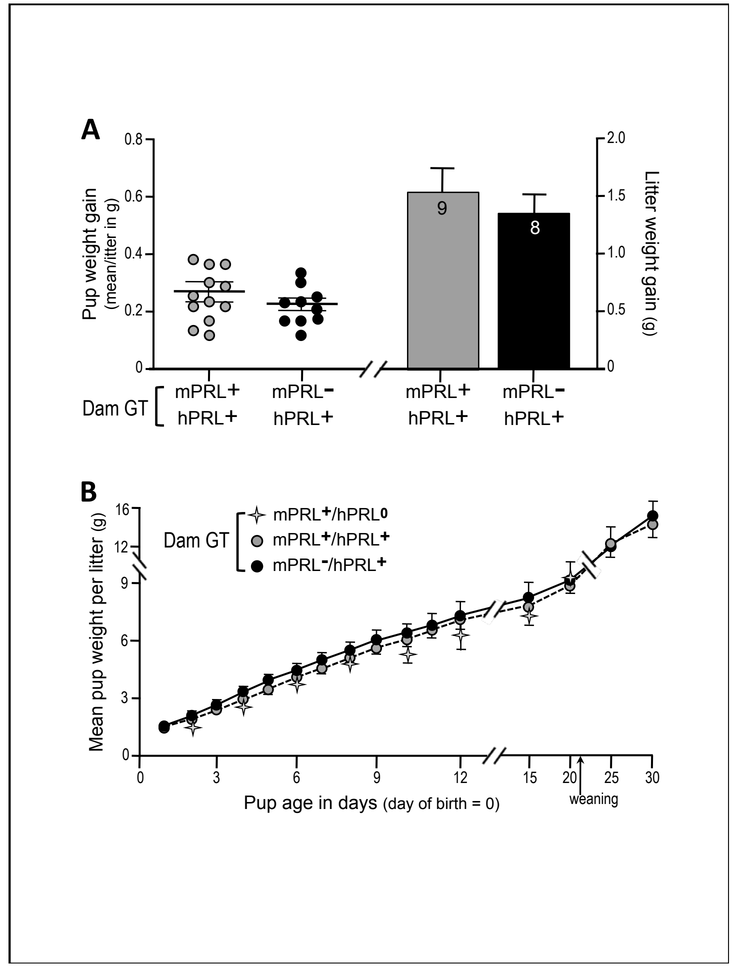

A
B
vehicle
E_2 (100 pM)
ICI
Human PRL (ng/10^5 cells)
Mouse PRL (ng/10^5 cells)
30
20
10
0
40
30
20
10
0
**
***
**
**
***
**
Male
Female
Male
Female

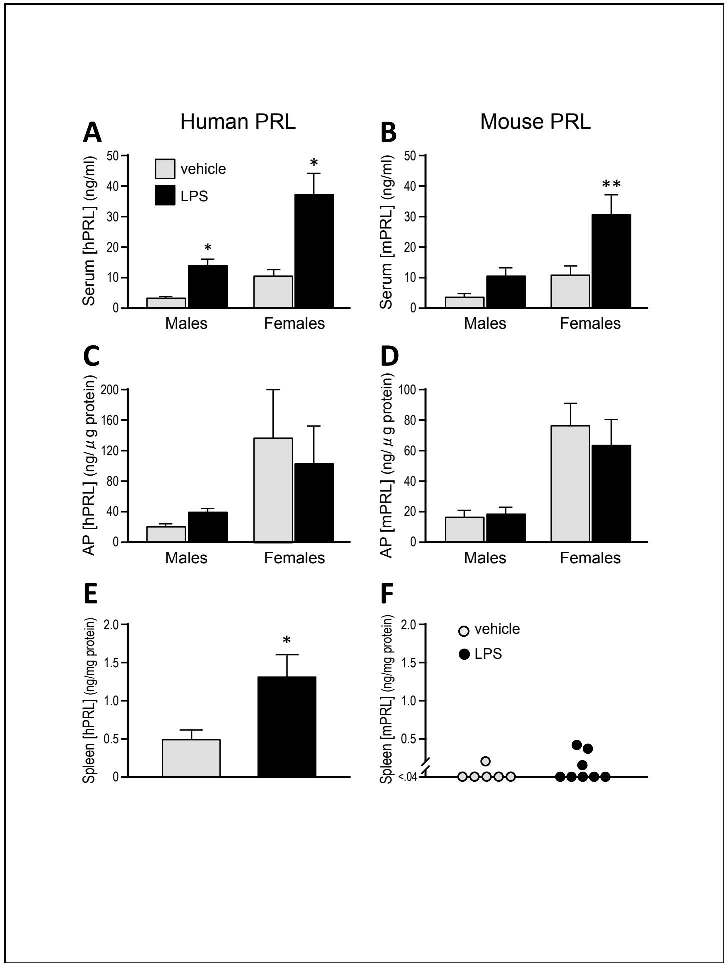

A
B
vehicle
Brx
Serum [hPRL] (ng/ml)
Serum [mPRL] (ng/ml)
25
20
15
10
5
0
25
20
15
10
5
0
*
*
Human PRL
Mouse PRL


A
vehicle
DA
TRH
Human PRL (ng/10^5 cells)
20
15
10
5
0
*
***
*
Male
Female
B
Mouse PRL (ng/10^5 cells)
25
20
15
10
5
0
*
***
*
Male
Female

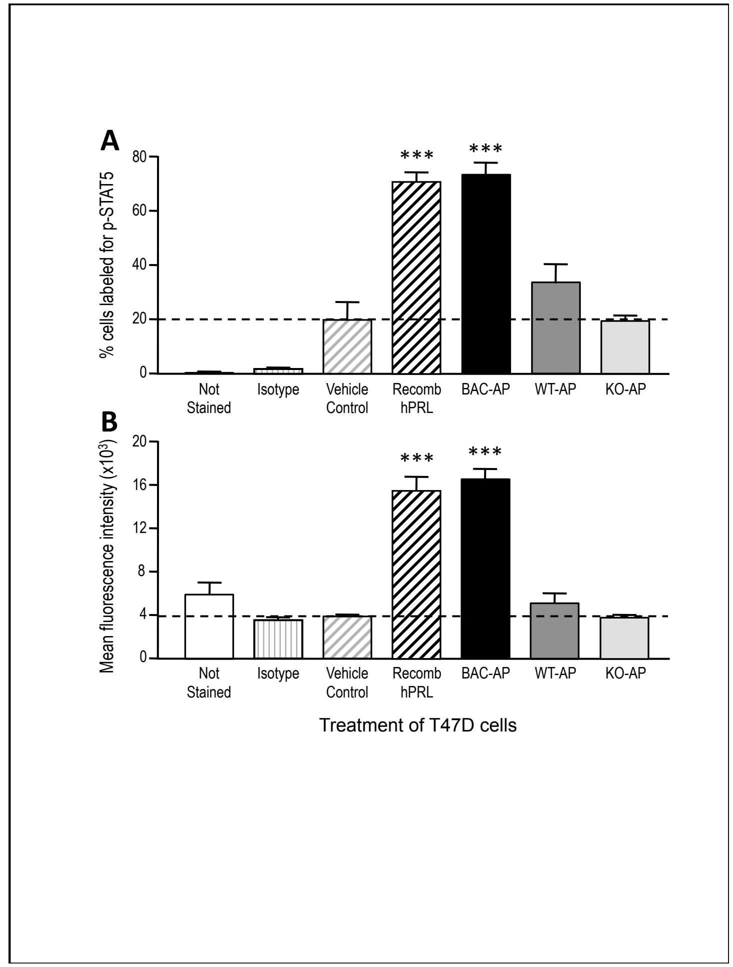

A
B
C
D
mPRL+/hPRL0
mPRL+/hPRL+
mPRL-/hPRL0
mPRL-/hPRL+


A
Vaginal opening (postnatal day)
5
14
***
15
15
Pup GT
mPRL+ hPRL0
mPRL+ hPRL+
mPRL- hPRL0
mPRL- hPRL+
B
Time to Delivery (days after plug)
NA
C
Pups per litter
Dam GT
mPRL+ hPRL0
mPRL+ hPRL+
mPRL- hPRL0
mPRL- hPRL+

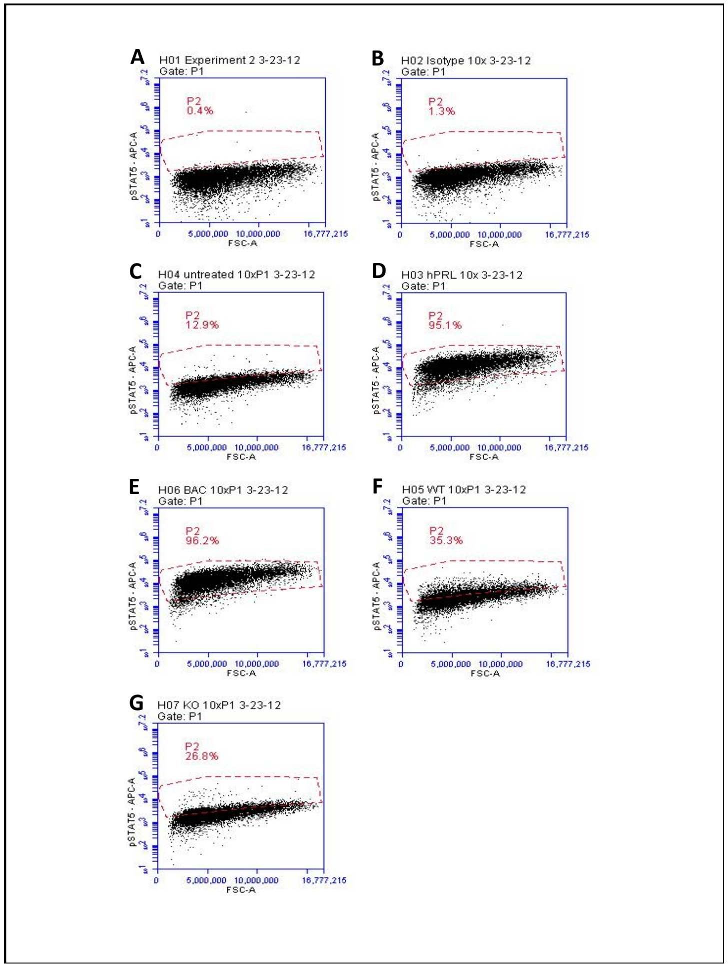

A
Pup weight gain (mean/litter in g)
Litter weight gain (g)
9
8
Dam GT
mPRL+ hPRL+
mPRL- hPRL+
mPRL+ hPRL+
mPRL- hPRL+
B
Mean pup weight per litter (g)
Dam GT
mPRL+/hPRL0
mPRL+/hPRL+
mPRL-/hPRL+
Pup age in days (day of birth = 0)
weaning



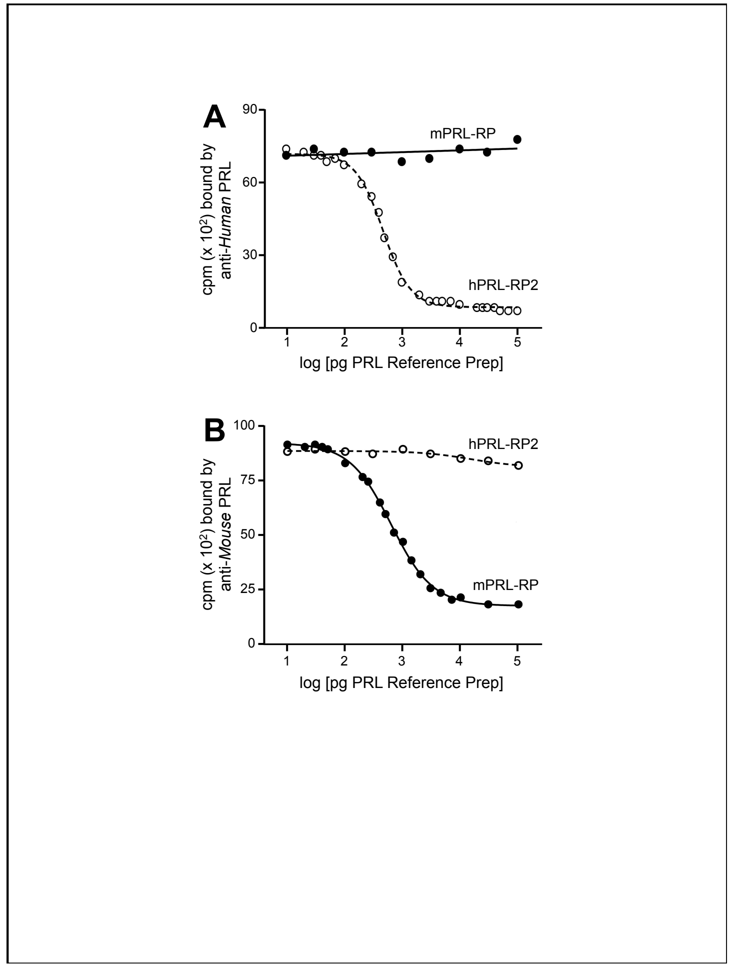
Human PRL
Mouse PRL
A
B
C
D
E
F
vehicle
LPS
Serum [hPRL] (ng/ml)
Serum [mPRL] (ng/ml)
AP [hPRL] (ng/ μg protein)
AP [mPRL] (ng/ μg protein)
Spleen [hPRL] (ng/mg protein)
Spleen [mPRL] (ng/mg protein)
Males
Females



A
B
% cells labeled for p-STAT5
Mean fluorescence intensity (x10³)
Not Stained
Isotype
Vehicle Control
Recomb hPRL
BAC-AP
WT-AP
KO-AP
Treatment of T47D cells



A H01 Experiment 2 3-23-12
Gate: P1
P2
0.4%
B H02 Isotype 10x 3-23-12
Gate: P1
P2
1.3%
C H04 untreated 10xP1 3-23-12
Gate: P1
P2
12.9%
D H03 hPRL 10x 3-23-12
Gate: P1
P2
95.1%
E H06 BAC 10xP1 3-23-12
Gate: P1
P2
96.2%
F H05 WT 10xP1 3-23-12
Gate: P1
P2
35.3%
G H07 KO 10xP1 3-23-12
Gate: P1
P2
26.8%
pSTAT5 - APC-A
FSC-A
0
5,000,000
10,000,000
16,777,215



A
cpm (x 10²) bound by anti-*Human* PRL
mPRL-RP
hPRL-RP2
log [pg PRL Reference Prep]
B
cpm (x 10²) bound by anti-*Mouse* PRL
hPRL-RP2
mPRL-RP
log [pg PRL Reference Prep]

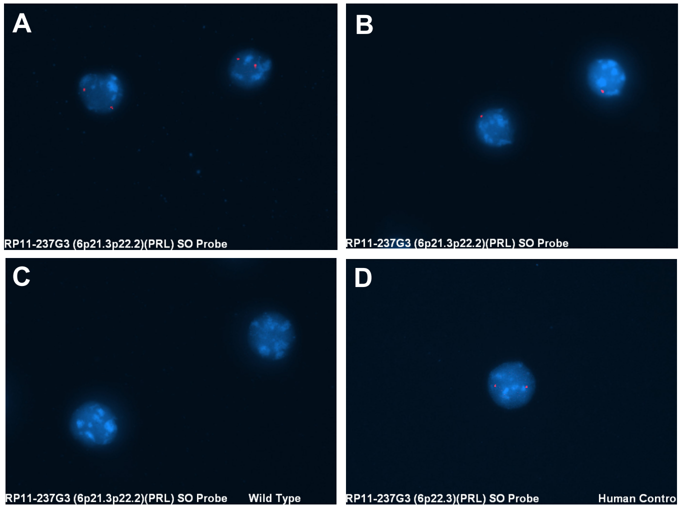


Table 1

Lab ID #	Sample ID	Sample Info	No. of Cells Analyzed	Signal Pattern
ZO-12-0009	H406	wild-type	100	No probe signals
ZO-12-0010	H344	BAC-h8 line	100	One probe signal
ZO-12-0011	H457	BAC-h30 line	100	Two probes signals